THE BRITISH JOURNAL OF EXPERIMENTAL PATHOLOGY

VOL. X OCTOBER, 1929 No. 5

THE ACTION OF AUSTRALIAN SNAKE VENOMS ON PLAIN MUSCLE.

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Forwarded for publication June 22nd, 1929.

THE venoms of some of the Australian elapines have a powerful stimulant action upon plain muscle *in vitro*, of a type which does not appear to have been described previously either for snake venoms or for any other substance. This effect appears to be wholly different from that of the toxins of *B. welchii* and of *Vibrion septique* observed by Buttle and Trevan (1928). Contraction of plain muscle caused by these bacterial toxins has a long latent period. The plain muscle, having once contracted maximally, cannot respond either to these toxins or to other stimuli for some time, though it recovers after sufficient washing in oxygenated Ringer's solution. The reaction is specific, and can be used for titration of antitoxin. Finally the toxins are unstable, and it is necessary to control the bubbling rate of oxygen or air very carefully in order to elicit the effect with small doses.

The effect of venoms on plain muscle described here resembles closely the anaphylactic response of sensitive plain muscle. When the muscle has responded to one venom it will not respond to the same or any other venom of the group (except to larger doses), but its reaction to other stimuli is unaffected. Having once been thoroughly desensitized with snake venom it does not recover its sensitiveness. The reaction has a latent period of the same order as that of the anaphylactic response of sensitive plain muscle, and by the use of a number of small doses of venom it is possible to desensitize the muscle without ever provoking a response. Experiments with antivenine are unfavourable to the use of this reaction for its titration.

Gunn and Heathcote (1921) studied the effect of cobra venom on the isolated heart, blood-vessels and intestine of the rabbit and cat. The type of contraction obtained with this venom appears from their experiments and from the experiments later recorded in this paper to be different from that of the venoms of the Australian elapines.

* This research was carried out under a grant from the Department of Health of the Commonwealth Government.

METHODS.

For most of the experiments recorded here I have used uterine horns of virgin guinea-pigs of 200 to 250 gm. weight suspended in a bath of 30 to 50 c.c. capacity containing oxygenated Ringer's solution. For comparison of different venoms or of mixtures of venom and antivenine the reactions of the two horns of the same uterus have been used whenever possible, since these have approximately the same sensitiveness to snake venom, and since the variation in sensitiveness in the plain muscle from different guinea-pigs is considerable.

The Ringer's solution used was "anaphylactic Ringer" (Dale, 1912), maintained at a temperature of 37·5° C. and at a pH of about 7·4 by methods earlier described (Kellaway, 1928). In a few experiments the uteri of virgin rats of 100 gm. weight were used, and in others strips of rabbit uterus—which were kept on ice till needed for periods of 2 or 3 days. For the rabbit uterus experiments the percentage of calcium chloride in the Ringer's solution was 0·024 instead of 0·012.

RESULTS.

(i) *The Type of Reaction.*

The type of reaction obtained with the guinea-pig's uterus is well shown in Fig. 1. After testing the sensitiveness of the uterine horn, which reacted well though not maximally with a concentration of 1 in 25 millions of histamine, a maximal response was obtained with 1 in a million of dry tiger snake (*Notechis scutatus*) venom. After changing the Ringer's solution the uterus failed to react with tiger-snake venom in the same concentration, but without washing out the bath a maximal response was obtained with 1 in 25 millions of histamine. This experiment is typical, though it does not represent the limits of activity of this, the most active of the venoms studied. After eliciting a maximal reaction, a second maximal reaction may often be obtained with a dose five or ten times as large, but as a rule no contraction, or at most a very small one, results when the first dose of the same venom or an equivalent stimulant dose (not necessarily the same amount) of another venom of the same group is added to the bath. Fig. 1 also illustrates a further characteristic feature of the reaction—treatment with venom, even without changing the Ringer's solution, failing to affect the subsequent response of the plain muscle

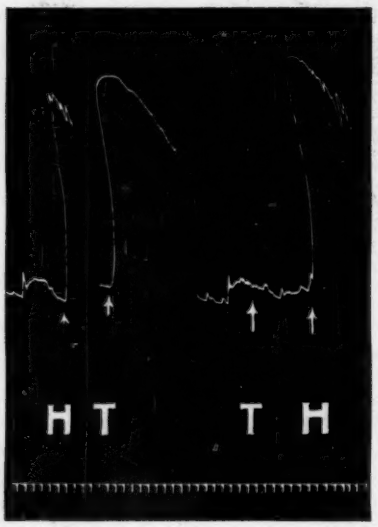


FIG. 1.—Reactions of one horn of the isolated uterus of a virgin guinea-pig suspended in a bath of 50 c.c. of oxygenated Ringer's solution. At H, 0·002 mgm. histamine; at T, 0·05 mgm. of tiger-snake venom added to the bath. Time in half minutes

to a small dose of histamine. This is true even when much higher concentrations (1 in 50,000) of the Australian elapine venoms are used.

In addition to its resemblance to the anaphylactic reaction of sensitive plain muscle in its latent interval and in the partial or complete desensitization which follows reaction, still another point of resemblance is seen in desensitization to snake venom by repeated doses of venom too small to cause any contraction of the uterus, so that after several small doses, a dose many times in excess of the minimal effective dose (M.E.D.) may be tolerated without a trace of reaction. This is illustrated in Fig. 2. This uterus was not as sensitive as

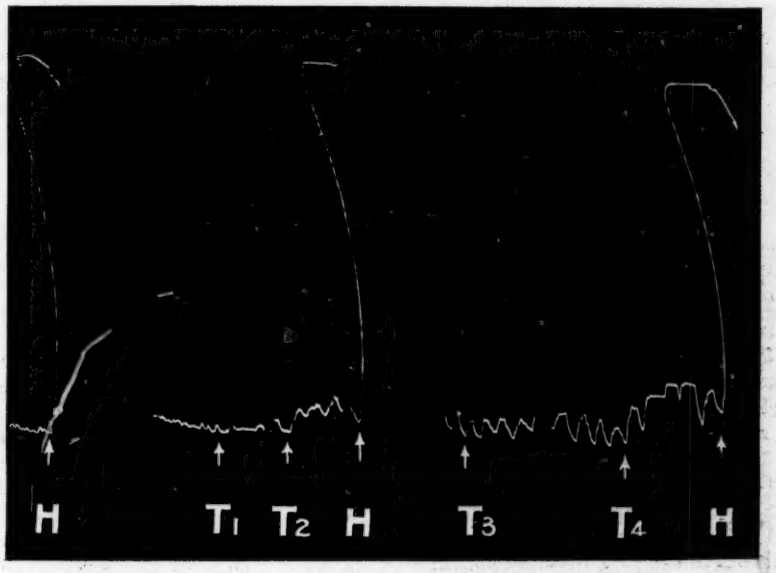
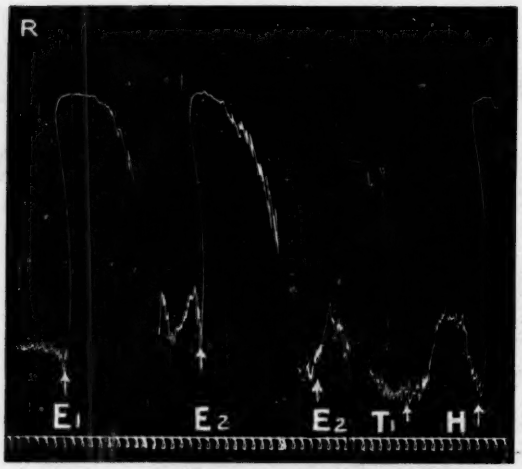


FIG 2.—Reactions of one horn of the isolated uterus of a virgin guinea-pig suspended in a bath of 50 c.c. of oxygenated Ringer's solution. At H, 0·002 mgm. histamine; at T_1, 0·002 mgm. of tiger-snake venom; at T_2, 0·01 mgm.; at T_3, 0·02 mgm.; at T_4, 0·2 mgm. of tiger-snake venom added to the bath. Drum at the same rate as in Fig. 1.

usual to snake venom, and should have given a reaction with the initial dose of venom. It reacted well to histamine, but after successive doses of tiger-snake venom of 0·002 mgm., 0·01 mgm. and 0·02 mgm., a dose of 0·2 mgm. caused hardly any response. The second horn gave a maximal response with 0·01 mgm. of tiger-snake venom. The bath was of 50 c.c. capacity, and the speed of the drum was that in the previous figure in which the time-marker recorded $\frac{1}{2}$-minute intervals. Since the sensitiveness of the muscle can be reduced by contact with traces of venom, great care must be taken in washing out the bath many times between the experiments just as in anaphylactic experiments.

Concentrations of snake venom too small to cause a maximal response give rise to smaller stepped reactions and a definite lengthening of the latent interval



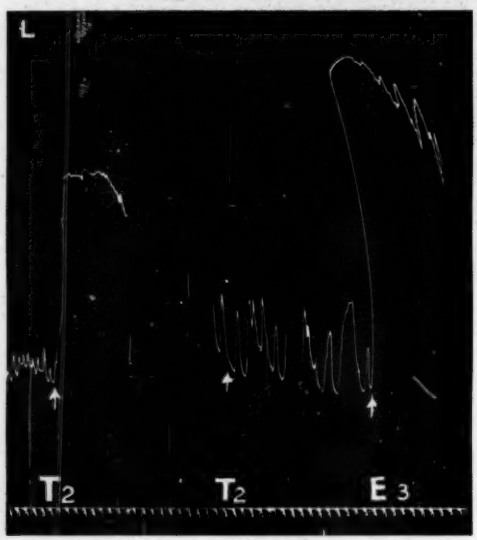


FIG. 3.—Reactions of the horns (R and L) of the uterus of a guinea-pig sensitized 22 days before by the subcutaneous injection of 5 mgm. of hen egg albumin. The capacity of the bath was of 30 c.c. At E_1, 0·05 mgm. of hen egg albumin; at E_2, 1 mgm. of egg albumin; at E_3, 0·01 mgm. of egg albumin; at T_1, 0·005 mgm. of tiger-snake venom; at T_2, 0·1 mgm. of tiger-snake venom and at H, 0·004 mgm. of histamine added to the bath. Time in minutes.

is observed, though its duration is never as great as that of the latent intervals which precede reaction with *Vibrion septique* toxin. A fallacy which has to be carefully guarded against in these experiments is the presence of "natural rhythm" in the uterine strip. In such cases, in which a large contraction takes place naturally every few minutes, error may easily arise, and unfortunately careful exclusion of rutting uteri by examination of the external genitalia (Stockard and Pampanicolau, 1919) does not wholly exclude this possibility.

The very close similarity which exists between the phenomenon under consideration and the anaphylactic reaction of sensitive plain muscle raised the question as to whether a hypothetical stimulant substance produced in the sensitive uterus by its specific anaphylactic antigen might not be identical with the stimulant substance present in the snake venoms. That this is not the case is shown by the repeatedly demonstrated fact, illustrated in Fig. 3, that the plain muscle of a virgin guinea-pig which has been sensitized by the injection of a foreign protein still gives the typical anaphylactic response to that protein after it has been desensitized with snake venom. A number of experiments of this kind were made, using the plain muscle from guinea-pigs killed 20 to 23 days after

the subcutaneous injection of 5 mgm. of crystalline egg albumin prepared for me by Miss Freeman. Maximal responses were regularly obtained with 0·05 mgm. of egg albumin in a bath of 30 c.c., and no obvious impairment of sensitiveness to egg albumin was noted after desensitization with snake venom. On the other hand, desensitization of the plain muscle with egg albumin seemed to make it somewhat less sensitive to snake venom.

The occurrence of a latent interval might be held to suggest that the reaction is an indirect one—the snake venoms acting either upon the muscle tissue itself or on other tissue present in the strip, and leading to the production of some stimulant substance. A similar argument has been applied, as hinted

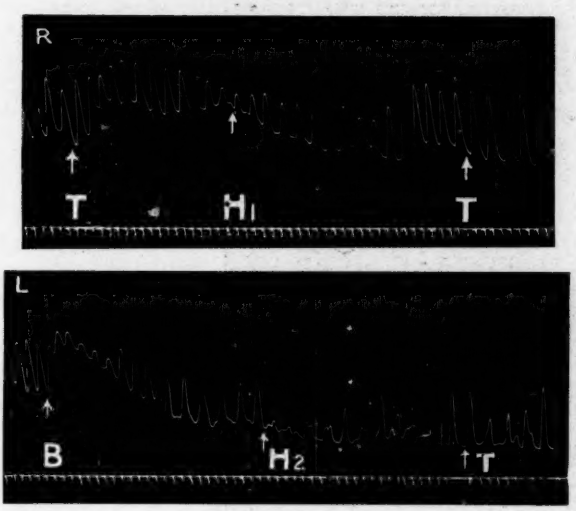


FIG. 4.—Reactions of the two horns (R and L) of the isolated uterus of a virgin rat suspended in a bath of 30 c.c. of oxygenated Ringer. At T, 2 mgm. of tiger-snake venom; at B, 2 mgm. of black-snake venom; at H_1, 0·1 mgm. of histamine; at H_2, 0·2 mgm. of histamine added to the bath. Time in minutes.

above, to explain the latent period in the anaphylactic reaction. An alternative explanation is afforded by the time taken for protein substances of relatively large molecular weight to diffuse into contact with the muscle-cells. It might be anticipated that, if this were the explanation, the latent period with snake venom would be less than that observed in the anaphylactic reaction, since, as will be seen later, most of the stimulant activity is present in the proteose fraction of the venom. The stimulant principle of the venom is not readily filterable through pyroxylin filters impermeable to hæmoglobin or to egg albumin at pressures of 300 to 400 mm. of mercury for periods of some hours. It is, however, possible that adsorption to coagulable proteins of larger molecular weight may explain both the slow ultra-filtration and the fact that the latent period observed appears to be of the same order as in the anaphylactic reaction,

though no very accurate observations have been made on this last point. The reaction of the plain muscle to snake venom might conceivably be due to the production of histamine or some similar substance by the action of venom on the tissue and the latent interval might be so explained. That the reaction is not due to the formation of histamine is shown by the fact that the uterus of the virgin rat regularly contracts with snake venom and, as is well known, relaxes with histamine. Fig. 4 shows two typical examples of this effect. It will be observed that much higher concentrations of venom are required to cause contraction in the uterus of the rat than in that of the guinea-pig, and here—the bath used being one of 30 c.c.—a concentration of 1 in 15,000 failed to cause a maximal response. In other respects the plain muscle of the two animals behaves similarly, that of the rat failing to react to a second dose of the same or of another venom. The characteristic relaxation with histamine is seen in concentrations of 1 in 300,000 and 1 in 150,000.

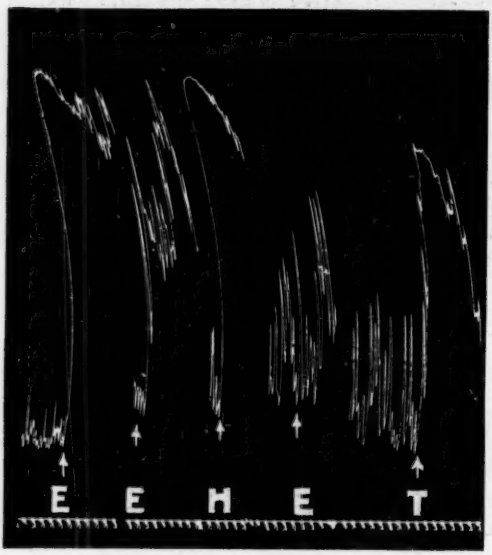


FIG. 5.—Reactions of a horn of the uterus of a virgin guinea-pig suspended in a bath of 30 c.c. of oxygenated Ringer's solution. At E, 0·4 mgm. of ergotoxin dissolved in 0·01 N NaOH in saline; at H, 0·01 mgm. of histamine; at T, 0·02 mgm. of tiger-snake venom added to the bath. Time in minutes.

Experiments with ergotoxin suggest that the action of the stimulant principle is a direct one upon the muscle itself and not indirect upon the nerve-endings. The uterus of the guinea-pig which has been treated repeatedly with doses of ergotoxin till it no longer responds to that drug still gives a typical reaction with a dose of snake venom somewhat greater than the M.E.D. (minimal effective dose), and on the other hand after desensitization with venom the reaction to ergotoxin is apparently unimpaired. There are difficulties in demonstrating these last statements satisfactorily, since to get the drug into solution moderately strong alkali is used, and this causes great increase in the rhythm of the plain muscle. I have found it satisfactory to dissolve 1 mgm. of ergotoxin in 1·0 c.c. of 0·01 N NaOH in physiological saline. The ergotoxin action can be controlled by ascertaining the effect of change in pH alone by the addition of a corresponding amount of 0·01 N NaOH to the bath. In the experiment recorded in Fig. 5, the second horn gave a small contraction and a large increase in rhythm when 0·4 c.c. of 0·01 N NaOH in saline was added to the bath, but after washing out, a maximal response similar to that figured was obtained with 0·4 mgm. of ergotoxin. This concentration

appeared to be sufficient to desensitize the plain muscle to the further action of ergotoxin. And after two further doses of ergotoxin a moderate response

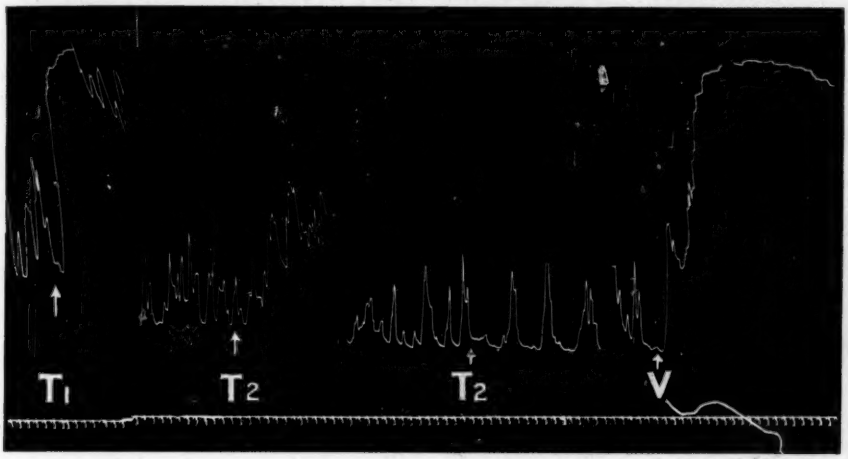


FIG. 6.—Reactions of a piece of the uterus of a virgin rabbit suspended in a bath of 30 c.c. of oxygenated Ringer's solution. At T_1, 0·5 mgm. of tiger-snake venom; at T_2, 1 mgm. of tiger-snake venom; and at V, 20 mgm. of *Vibrion septique* toxin added to the bath. Time in minutes.

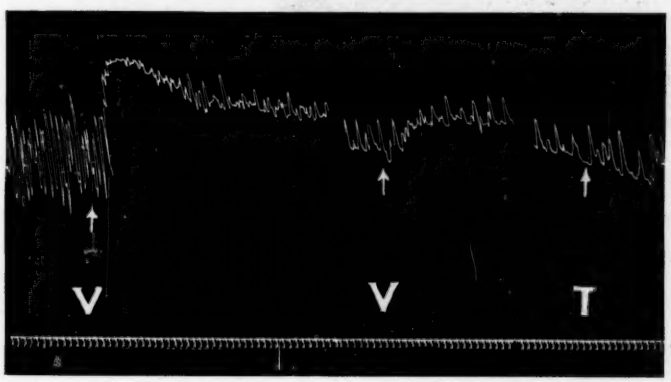


FIG. 7.—Reaction of another piece of the same uterus as in Fig. 6. At V, 18 mgm. of *Vibrion septique* toxin, and at T, 1 mgm. of tiger-snake venom added to the bath. Time in minutes.

was obtained with 0·02 mgm. of tiger snake venom. I have repeated these experiments with neutral solutions of ergotoxin prepared for me by Mr. H. F. Holden, in which the drug is held in solution by a protective colloid (gelatine 0·8%), which itself has no pharmacological action. Even in these solutions the

drug causes a very marked increase in the rhythm of the uterus. There was some impairment in the response to 0·005 mgm. of tiger-snake venom, a stepped half maximal response being obtained after treating plain muscle with 4 successive doses of 0·4 mgm. of ergotoxin in a bath of 30 c.c. If the action of ergotoxin is upon the end-plates in the muscle and not upon the muscle-cells themselves, it is reasonable to infer that the snake venom acts upon the muscle directly. Alternatively it might be held that, as is the case with nerve-endings in vascular plain muscle, some only of the nerve-endings are paralyzed by ergotoxin.

Apart from the obvious differences already indicated, clear evidence of the dissimilarity of the stimulant substances in these venoms from that present in the toxin of *Vibrion septique* is afforded by desensitizing the uterus with a venom and subsequently eliciting a maximal reaction with *Vibrion septique* toxin (Fig. 6). On the other hand, for some time after the administration of *Vibrion septique* toxin the reaction with the snake venoms cannot be elicited (Fig. 7). In these last experiments I used strips of rabbit uterus, since the uterus of the guinea-pig proved unsuitable, remaining contracted for too long a time after treatment with *Vibrion septique* toxin.

(ii) *The Venoms which Possess Stimulant Activity.*

All the Australian elapine venoms which I have so far tested give in various concentrations the typical reaction described. These include the venoms of the following reptiles:

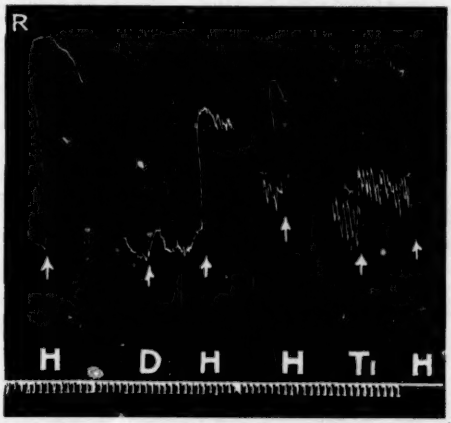
Notechis scutatus (tiger snake).
Acanthophis antarcticus (death adder).
Denisonia superba (copper head).
Pseudechis porphyriacus (black snake).
Pseudechis guttatus (a rare speckled snake somewhat akin to the last).
Oxyuranus maclennani (a large and rare snake described by Kinghorn, 1923).
Domansia textilis (a common variety of brown snake).

The venoms from two other rare snakes not yet certainly identified also give typical reactions.

The venoms from these snakes do not all show the same degree of activity, and, as will be seen, the variation of stimulant activity for guinea-pig plain muscle is only roughly paralleled by their toxicity for the animal itself.

I have compared the stimulant activity of the venoms of the tiger snake, copper head, death adder and black snake. For this purpose it is best to determine the minimal effective dose (M.E.D.), since even very large doses fail to cause as complete a contraction as does histamine in a concentration of 1 in 10 or 1 in 20 millions. The venoms of the tiger snake and copper head are most active, causing reactions in concentrations of 1 in 30 to 1 in 60 millions. That of the death adder has rather more than one-tenth of this activity, giving large reactions in concentrations of 1 in 3 to 1 in 6 millions. The venom of the black snake is less active still, giving similar reactions in concentrations of 1 in 500,000. Fairley's (1929) studies on sheep, and my

own (1929) on horses, monkeys, cats, rabbits, guinea-pigs, rats and mice, indicate that these venoms may be placed in the following order of toxicity: (1) tiger snake, (2) death adder, (3) copper head, and (4) black snake. For



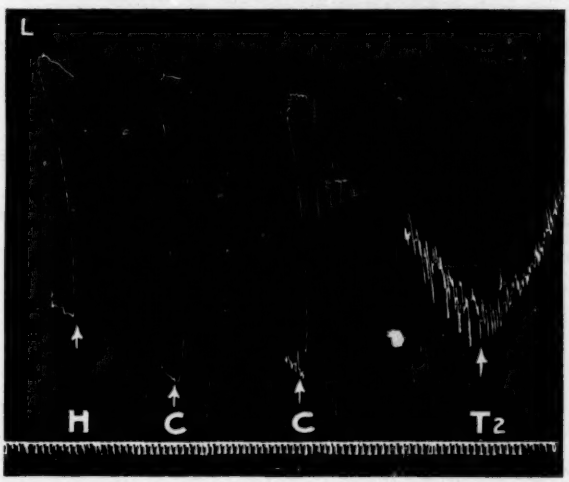


FIG. 8.—Reactions of the horns (R and L) of the uterus of a virgin guinea pig suspended in a bath of 30 c.c. of oxygenated Ringer's solution. At H, 0·004 mgm. of histamine; at D, 20 mgm. of daboia venom; at T_1, 1 mgm. of tiger-snake venom; at T_2, 0·1 mgm. of tiger-snake venom; and at C, 0·5 mgm. of cobra venom added to the bath. Time in minutes.

the guinea-pig of about 300 grm. weight, the certainly lethal doses subcutaneously are for tiger snake venom 0·002 mgm., for copper head 0·006, for death adder 0·015, and for black snake venom approximately 0·16 mgm per 100 gm. weight.

The only two venoms of snakes other than those occurring in Australia which I have had the opportunity of investigating are those of the cobra (*Naia tripudians*) and of the daboia (*Vipera russelli*). Of the former, larger doses are required to cause contraction of the plain muscle than of the more potent Australian venoms, and several repeated doses frequently give about the same amount of contraction. The results in different experiments were not, however, very regular, and in some the type of reaction was similar to that given by the Australian venoms, a second dose causing a smaller reaction than the first. After treatment with repeated doses of cobra venom, the uterus was generally desensitized, at least to some extent, to the Australian elapine venoms.

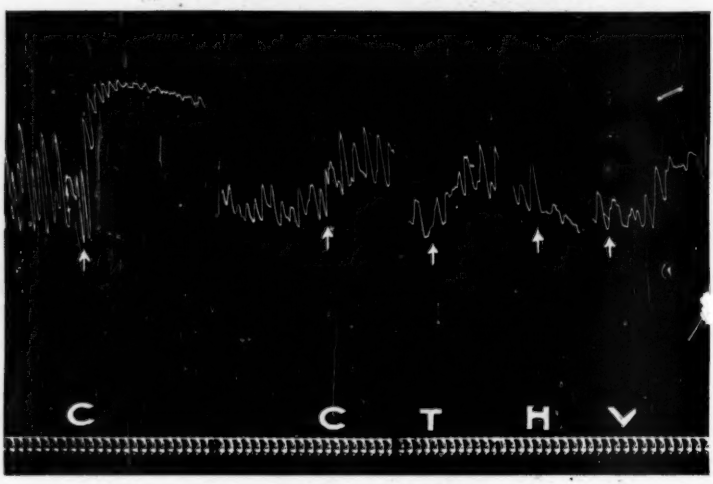


FIG. 9.—Reactions of one horn of the isolated uterus of a virgin rat suspended in a bath of 30 c.c. of oxygenated Ringer's solution. At C, 0·4 mgm. of cobra venom; at T, 0·2 mgm. of tiger-snake venom; at H, 0·2 mgm. of histamine; and at V, 20 mgm. of *Vibrion septique* toxin added to the bath. Time in minutes.

On the other hand, good reactions were obtained with cobra venom after desensitization with Australian venoms.

It seemed likely that the stimulant substance in cobra venom was different from those in the Australian venoms, but like the latter, it caused a powerful contraction of the uterus of the virgin rat (Fig. 9). The stimulant activity was not therefore due to histamine. In this experiment after treatment with two successive doses of cobra venom the rat plain muscle showed diminished sensitiveness to an Australian venom, and to some extent also to *Vibrion septique* toxin.

Experiments with daboia venom also gave somewhat irregular results. In concentrations of 1 in 25,000 no contraction was produced, but the uterus was desensitized to the action of Australian venoms. In one experiment, using daboia venom in a concentration of 1 in 3000 a submaximal contraction of the

uterus was obtained. A second dose gave no contraction, and finally tiger-snake venom caused no contraction in a concentration of 1 in 60,000. In other experiments 1 in 4000 and 1 in 1500 of daboia venom failed to cause any contraction, but the plain muscle was damaged by these large doses, and though it failed to react with moderate concentrations of Australian venoms, it also contracted poorly or not at all with histamine in a concentration of 1 in 25 millions. The results in a similar experiment are figured (Fig. 8).

Difficulty in interpreting these results is due to the fact that with the high concentrations of these venoms necessary to produce an effect there is some damage to the reactivity of the muscle. It is difficult on the evidence I have obtained to decide whether the stimulant substance in cobra venom can exhaust the "receptors" of the plain muscle-cells so that the Australian venoms can no longer produce their characteristic effect, and whether in the case of daboia there is present an amount of a stimulant substance too small to cause contraction but sufficient to use up the "venom receptors" of the muscle, or alternatively, whether the results are not wholly explainable in terms of damage to the muscle-cells. Whichever explanation of the experimental data is the true one, it is certain that these two venoms are quantitatively widely different from the Australian venoms under consideration.

(iii) *The Nature of the Stimulant Substance.*

This has by no means been completely explored, but certain preliminary studies have been carried out.

(a) *The Heat Stability of the Active Substance.*

A few experiments were carried out with the venom of the tiger snake (*Notechis scutatus*). The venom in a concentration of 0·01% in saline was heated to 92°–94°C. (in a boiling water-bath) at a pH of 7·6 for periods of 2, 4, 6, 8 and 10 minutes. Loss by evaporation was made up before testing the solution. After two minutes about 99% of the "thrombase" activity was destroyed as judged by intravenous injections in rabbits. That this active principle of tiger-snake venom, as well as that of black snake (*Pseudechis porphyriacus*) is readily destroyed by heat is well known from the classical experiments of Martin (1893 and 1905). The intravenous certainly lethal dose of tiger-snake venom for rabbits of the kind used is 0·002 mgm. per kilo (Kellaway, 1929). Rabbits receiving the venom heated for 2 minutes in amounts equivalent to 100 certainly lethal doses showed no immediate symptoms, but died with neurotoxic symptoms after about 7 hours. Those which received the equivalent of 25 certainly lethal doses of material heated 4 minutes and 6 minutes died with neurotoxic symptoms in 24 and 48 hours, and those which received the same equivalent doses of material heated for 8 and 10 minutes presented no symptoms and survived. None of the animals which died showed any trace of thrombosis. Rapid death with typical intravascular thrombosis was caused in rabbits injected with one certainly lethal dose of the unheated solution. These experiments gave no clue to the loss by heat of neurotoxic activity. In this experiment the stimulant action on plain muscle was very little impaired after 4 minutes,

about 50% of its activity was lost in 6 minutes, and about 80% after 8 and 10 minutes' heating. In another similar experiment at a pH of 6·5 less than half the stimulant activity was lost after 7 minutes, more than 80% after 12 minutes, and more than 90% after 32 minutes at 92° to 94°C. In 30 minutes at pH 6·5 and 73°C. about 80% of the stimulant activity was lost.

The results of a further experiment in which Miss Freeman kindly carried out the heating and readjustment of the venom solutions for me are given in detail here (Protocol 1).

The loss in neurotoxic activity estimated by subcutaneous injections in guinea-pigs was compared with that of the muscle stimulant activity.

PROTOCOL 1.—*The Effect of Heat on the Neurotoxic and Muscle-Stimulant Activities of Tiger-Snake Venom.*

The venom in 1% solution in saline was heated at 92° to 93° C. for 5, 10 and 20 minutes. The C.L.D. of this venom is 0·002 mgm. per 100 gm. for guinea-pigs of the weight used.

EFFECT ON NEUROTOXIC ACTIVITY.

Venom.	Number of guinea-pigs injected.	Equivalent in C.L.Ds	Result.
Unheated	4	1	Two died in 39 and 2 in 53 hours.
Heated 5 mins.	2	6	No symptoms, survived.
,, 5 ,,	2	4	,, ,,
,, 5 ,,	4	2	,, ,,
,, 10 ,,	2	15	Both died in less than 48 hours.
,, 10 ,,	2	10	No symptoms; survived.
,, 20 ,,	2	30	,, ,,
,, 20 ,,	2	10	,, ,,

EFFECT ON MUSCLE-STIMULANT ACTIVITY.

Right horn.			Left horn.		
Venom.	Dose	Reaction.	Venom.	Dose.	Reaction.
Unheated	0·002	Nearly maximal	Heated 20 mins.	0·06	Stepped, submaximal.
,,	0·001	Do.	,, 10 ,,	0·01	Immediate, nearly maximal.
,,	0·001	,,	,, 10 ,,	0·005	More than ½ maximal.
—	—	—	,, 10 ,,	0·002	Small, stepped.
Unheated	0·001	⅔ maximal	,, 5 ,,	0·002	Delayed, stepped, less than ½ maximal.

From these plain-muscle experiments it appears that in 5 minutes more than 50% of the stimulant activity has been destroyed, in 10 minutes more than 80% of this activity has been lost, and that in 20 minutes more than 90% of the activity has been destroyed. The results of the injection experiments in guinea-pigs show that after 5 minutes more than 80% of the neurotoxic

activity is lost, after 10 minutes at least 90%, and after 20 minutes' heating about 96% of the neurotoxic activity is destroyed. Whereas, therefore, the stimulant activity of tiger-snake venom is much more stable than the coagulant activity, the loss by heat of the stimulant and neurotoxic activities is parallel within the limits of accuracy of the methods used to assay them. The apparent dissociation of the coagulant activity of tiger-snake venom from the stimulant activity is not surprising, since copper-head venom, which has been shown to possess no coagulant activity (Fairley, 1929; Kellaway, 1929), is nearly as active as is tiger in stimulant activity for the plain muscle of the guinea-pig.

With Miss Freeman I have also investigated the effect of heat upon death-adder venom. After 5 minutes' heating at 92° to 93° the loss of toxicity for the guinea-pig was not so great as with tiger-snake venom. Of guinea-pigs receiving the equivalent of 1·5 and 2 lethal doses of venom heated for 5 minutes, 1 died and all showed severe symptoms. Of guinea-pigs receiving the equivalent of 8, 12 and 15 certainly lethal doses of venom heated for 20 minutes, all died in less than 24 hours, but those receiving the equivalent of 3 and 5 certainly lethal doses survived without symptoms. Finally, guinea-pigs injected with the equivalent of 20 certainly lethal doses and 15 certainly lethal doses of venom heated for 30 minutes died in less than 24 hours, and those receiving 4 and 5 certainly lethal doses showed no symptoms and survived.

The effect of heat on the muscle-stimulant activity was estimated as follows:

Right horn.			Left horn.		
Venom.	Dose (mgm.).	Reaction.	Venom.	Dose (mgm.).	Reaction.
Heated 30 mins.	0·3	Very small	Heated 20 mins..	0·3	More than ½ maximal.
Unheated . .	0·01	Nearly maximal	„ 10 „ .	0·12	¾ maximal.
Heated 5 mins. .	0·06	Do.	„ 10 „ .	0·03	*Nil.*

These results, though much too few for the accurate titration of the various solutions of venom, suggest that after 10 minutes' heating between 70% and 90% of the muscle-stimulant activity is lost, after 20 minutes about 95%, and after 30 minutes about 99% of the activity has been destroyed. The loss of toxicity, though more animals were needed to determine it accurately, roughly corresponded with the loss of stimulant activity. Experiments with copper-head venom also showed loss of stimulant and neurotoxic activity on heating. The stimulant substances in the venoms, like their neurotoxic principles, are thermolabile, though less so than the "thrombase" of tiger-snake venom.

Experiments with Miss Freeman on the fractionation of the venoms of the copper head and death adder suggest that the stimulant activity of these venoms, like the neurotoxic activity, resides principally in the proteose fraction. In these experiments, in which a 1% solution of venom was used, the coagulable protein was removed from solution by heating in the water-bath for 4 minutes. The coagulum was centrifuged down and repeatedly washed, the washings being added to the supernatant solution. From this the primary proteoses

were removed by half saturation with ammonium sulphate, and finally the secondary proteoses were precipitated by full saturation with ammonium sulphate and removed by filtration through a No. 40 Whatman filter. The fractions were all dissolved in saline so as to contain the equivalent of 1 mgm. of the venom per c.c. The control solution was made up from the original stock of freshly-made 1% solution from which 1·5 c.c. had been taken for the experiment. A trace of alkali had to be added to the coagulable protein fraction to bring it into solution, and therefore a further control was made up from the original solution by heating for the same time and then bringing the coagulable protein again into solution with alkali.

(b) *Results of Subcutaneous Injection into Guinea-Pigs.*

Of the *coagulable protein fraction*, the equivalent of 40 certainly lethal doses produced severe symptoms, but the animal recovered. The equivalent of 20 certainly lethal doses produced no symptoms. Of the *primary proteose fraction* doses equivalent to 15 and 20 certainly lethal doses were fatal in less than 24 hours, the equivalent of 4, 6 and 8 certainly lethal doses produced no symptoms, and of 10 certainly lethal doses only moderate symptoms. Of the *secondary proteose fraction* the equivalent of 2 certainly lethal doses produced no symptoms, but that of 4 and 6 certainly lethal doses killed regularly in a large series of animals. The heated control to which alkali had been added was regularly fatal in an equivalent of 2 certainly lethal doses.

Experiments with the isolated plain muscle of the virgin guinea-pig showed that whereas the *coagulable protein* from 4 mgm. of venom in a 30 c.c. bath gave no trace of reaction and failed to desensitize the uterus, the *primary proteose fraction* from 1 mgm. gave a moderate response, and the same dose of the *secondary proteose fraction* gave a nearly maximal response. Other experiments of this type gave similar results, and though it seemed likely that most of the stimulant and toxic activity resided in the proteose fraction, the loss which has been shown to occur on heating even for 4 minutes (at least 50%) and difficulties (probably related to adsorption) in the separation of the two proteose fractions make it almost impossible to get very efficient separation by this method.

Miss Freeman and I have also found that the muscle-stimulant principle of death-adder venom only passes with difficulty through pyroxylin filters made by Adair's (1915) method, and known to be impermeable to hæmoglobin and to egg albumin but permeable to erythro-dextrins. The separation of the coagulable proteins from the proteose fraction which can be effected in this way at low pressures, 300 to 400 mm. of mercury, is by no means quantitative. In one such experiment with death-adder venom the filtrate made up to the original volume of the solution gave a ½ maximal response with a dose equivalent to 0·5 mgm. of venom and a very small response with one-fifth of this amount. About 1% of the stimulant substance had therefore passed this filter in 11 hours at 0° C. and 300 mm. of mercury pressure. To effect a more complete separation of the coagulable protein from the proteoses, recourse will probably have to be had to ultra-filtration at high pressures demonstrated by Martin (1896) in the case of black-snake venom.

(iv) *The Neutralization of the Active Principle by Antivenines.*

Through the courtesy of Dr. F. G. Morgan, Director of the Laboratories Division of the Commonwealth Department of Health, I was able to use for these tests low potency antivenines—sera taken from horses during the course of immunization against the venoms of the tiger snake and death adder at the Commonwealth Serum Laboratories. I have compared the neutralizing value of these antivenines against the venoms of the tiger snake, death adder and copper head using subcutaneous injection in guinea-pigs and tests on the isolated virgin uterus from animals of the same species. For the protection experiments the intravenous method could not be used, since tiger-snake venom, as is well known, has a powerful coagulant action, while the other two venoms have no such effect. The guinea-pig has been chosen for these tests, since its plain muscle has been used in the parallel tests with plain muscle. In the protection tests varying amounts of solutions of carefully dried venom have been added to 1 c.c. of antivenine, and allowed to stand for at least 1 hour at room temperature before being injected subcutaneously. To eliminate individual variation reliance has been placed on the number of certainly lethal doses neutralized. In the tests of tiger-snake antivenine against copper-head and death-adder venoms, and of death-adder antivenine against copper-head and death-adder venoms, groups of animals should have been used for injection, as the number of certainly lethal doses is too small. The number of certainly lethal doses has been calculated from those doses, which in the determination of the characteristics by subcutaneous injection in guinea-pigs weighing from 260 to 285 gm., killed from 95% to 100% in groups of 20 or more animals (Kellaway, 1929). Owing to shortage of animals I have not been able to keep the weights of the guinea-pigs used in the protection experiments to be described here as uniform as desired, and some of the irregularities arise from this source.

Animals of heavier body-weight have a lower certainly lethal dose per 100 gm. for these venoms, and this, together with the fact that the certainly lethal doses used are, in the case of tiger snake and copper head, only approximate, introduces a further source of error. If a more potent antivenine had been available it would have been better to vary the antivenine, use a fixed dose of each venom and determine the " characteristic " for each venom and antivenine. This was not possible with the amounts of antivenines and the number of guinea-pigs at my disposal.

In expressing the amounts of venom neutralized by 1 c.c. of antivenine, I have taken as approximate limits the lowest dose which appears to kill regularly minus the certainly lethal dose for one of the animals at this dose, and the highest dose which regularly protects minus the certainly lethal dose for one of the animals at this dose. Theoretically the amount which should be subtracted from both values should be the $L_+ - Lo$ dose for 1 c.c. of antivenine (a somewhat larger value than the certainly lethal dose). I have used the certainly lethal dose as a first approximation. To eliminate individual variation in the plain muscle experiments, I have relied on the comparison (wherever possible) of the reactions of two horns of the same uterus which are placed on the same line in the protocols which follow. The mixtures of 0·2 c.c. of antivenine with varying amounts of venom have also been

allowed to stand at room temperature for at least an hour before testing. The approximate limits of neutralization have been estimated using the same principle as in the protection tests, subtracting the minimal effective dose from the smallest dose causing a reaction and the largest dose which failed to do so.

No claim is made as to final accuracy as the number of experiments is too small, but the results given here are sufficient to substantiate my deductions, (1) that qualitatively these two antivenines are not strictly specific when their behaviour with the venoms of closely allied species is considered, and (2) that the use of the isolated plain muscle gives results widely different quantitatively from the protection tests.

PROTOCOL 2.—*Tiger-Snake Antivenine against Tiger-Snake Venom.*

(*a*) PROTECTION TESTS ON GUINEA-PIGS.

Weight of guinea-pig, gm.	Approximate number of C.L.D.'s.	Total weight of venom in mgm. + 1·0 c.c. anti-venine	Result.
367	18	·132	Died in less than 24 hours.
244	24·5	·120	Moderate symptoms; recovery.
379	16	·121	Died in less than 24 hours.
255	23·5	·120	Died in 29 hours.
285	20	·110	No symptoms ; survived.
277	20	·110	,, ,,
265	19	·100	,, ,,
295	17	·100	,, ,,
252	18	·090	,, ,,
270	16·5	·090	,, ,,

The certainly lethal dose (C.L.D.) of this venom for guinea-pigs of the weight used is 0·002 mgm. per 100 gm. (Kellaway, 1929). The antivenine neutralizes between 0·110–·005 = 0·105 mgm. and 0·120–·005 = 0·115 mgm. per c.c. The estimated number of C.L.D.'s for the two heavy guinea-pigs is too low, as increase in body-weight lowers the C.L.D. per 100 gm.

(*b*) TESTS ON THE ISOLATED PLAIN MUSCLE.

Right horn.			Left horn.	
Antivenine.	Venom.	Reaction.	Venom.	Reaction.
0·2	·003	*Nil*	·003 (with 0·2 horse-serum)	Maximal.
0·2	·006	,,	·0015 (with 0·2 horse-serum)	⅔ maximal.
0·2	·008	Very small, stepped	·008 (with 0·2 death-adder antivenine)	*Nil.*
0·2	·009	Nearly maximal, stepped	·001	½ maximal.
0·2	·01	Small reaction	·012 (with 0·2 tiger-snake antivenine)	,,
0·2	·011	Nearly maximal	·001	More than ½ maximal.

The minimal effective dose of tiger-snake venom is about 0·0005. Between 0·008–0·0005 = 0·0075 mgm. and 0·009–0·0005 = 0·0085 mgm. is neutralized by 0·2 c.c. The antivenine neutralizes between 0·0375 and 0·0425 mgm. per c.c.

PROTOCOL 3.—*Tiger-Snake Antivenine against Copper-Head Venom.*

(*a*) PROTECTION TESTS ON GUINEA-PIGS.

Weight of guinea-pig, gm.	Approximate number of C.L.D.'s.	Weight of venom in mgm.	Result.
234	4	0·060	Died in less than 20 hours.
269	3	0·055	Died in 29 hours.
254	3	0·050	Died in less than 24 hours.
274	2	0·040	No symptoms, survived.
270	2	0·035	,, ,,
248	2	0·030	Died in 48 hours.
302	1·5	0·031	No symptoms, survived.
232	1·5	0·024	,, ,,

The certainly lethal dose of this venom is 0·006, but some deaths occur with 0·0025 mgm. per 100 gm. (Kellaway, 1929). The antivenine neutralizes between 0·040–0·016 = 0·024 mgm. and 0·050–0·015 = 0·035 mgm. per c.c.

(*b*) TESTS ON THE ISOLATED PLAIN MUSCLE.

Right horn.			Left horn.		
Antivenine.	Venom.	Reaction.	Antivenine.	Venom.	Reaction.
0·2	0·0025	*Nil*	...	0·001	Maximal.
0·2	0·005	,,	...	0·0005	½ maximal.
0·2	0·0055	⅔ maximal	0·2	0·005	Very small, delayed
0·2	0·006	,,	0·2	0·01	Nearly maximal.

A minimal effective dose of copper-head venom is 0·0005 mgm. About 0·0050–0·0005 = 0·0045 mgm. is neutralized by 0·2 c.c. of tiger-snake antivenine. This antivenine neutralizes about 0·0225 mgm. per c.c.

PROTOCOL 4.—*Tiger-Snake Antivenine against Death-Adder Venom.*

(*a*) PROTECTION TESTS ON GUINEA-PIGS.

Weight of guinea-pig, gm.	Approximate number of C L.D.'s.	Weight of venom in mgm.	Result.
262	2	0·075	Died in less than 20 hours.
309	1·5	0·065	Slight symptoms; survived.
307	1·3	0·060	Died in less than 20 hours.
275	1·6	0·065	No symptoms; survived.
292	1·4	0·060	Slight symptoms; survived.
237	1·5	0·055	Died in less than 44 hours.
265	1·3	0·050	No symptoms; survived.
265	1·3	0·050	,, ,,

The certainly lethal dose of death-adder venom is 0·015 mgm. per 100 gm. (Kellaway, 1929), but deaths occur with doses less than 0·01 mgm. per 100 gm. The antivenine neutralizes about 0·065–0·046 = 0·019 mgm. per c.c.

(*b*) Tests on the Isolated Plain Muscle.

Right horn.			Left horn.		
Antivenine.	Venom.	Reaction.	Antivenine.	Venom.	Reaction.
0·2	0·01	*Nil*, not desensitized	0·2	0·012	*Nil*, not desensitized.
0·2	0·02	Do.	0·2	0·04	Nearly maximal.
0·2	0·05	Nearly maximal	0·2	0·04	,,
0·2	0·03	Less than ½ maximal	0·2	0·025	*Nil*, some desensitization.

The minimal effective dose is 0·005. Between 0·025–0·005 = 0·02 and 0·03–0·005 = 0·025 mgm. are neutralized by 0·2 c.c. This antivenine neutralizes between 0·1 and 0·125 mgm. per c.c.

Protocol 5.—*Death-Adder Antivenine against Copper-Head Venom.*

(*a*) Protection Tests on Guinea-Pigs.

Weight of guinea-pig, gm.	Approximate number of C.L.D.'s.	Weight of venom in mgm.	Result
285	4	0·065	Died in 44 hours.
247	4	0·060	Moderate symptoms ; survived.
247	3·5	0·055	,, Do.
237	3·5	0·050	Died in 44 hours.
240	3·5	0·050	Died in 48 hours.
235	3·5	0·050	Slight symptoms ; survived.
240	3·5	0·050	Died in 48 hours.
235	3	0·045	No symptoms ; survived.
234	3	0·040	,, ,,
264	2	0·036	,, ,,

The certainly lethal dose of copper head is 0·006 mgm. per 100 gm. The antivenine neutralizes between 0·045–0·014 = 0·031 mgm. and 0·050–0·014 = 0·036 mgm. per c.c.

(*b*) Tests on the Isolated Plain Muscle.

Right horn.			Left horn.		
Antivenine.	Venom.	Reaction.	Antivenine.	Venom.	Reaction.
0·2	0·002	*Nil*	...	0·001	More than ⅔ maximal.
0·2	0·003	,,	...	0·002	Do.
0·2	0·0035	,, not desensitized	0·2	0·004	½ maximal.
0·2	0·004	Nearly maximal	(0·2 horse-serum)	0·0025	Nearly maximal.
0·2	0·005	Do.	Do.	0·001	½ maximal.
...	...	...	,,	0·005	Nearly maximal.

The minimal effective dose of copper head is 0·0005. Between 0·0035–0·0005 = 0·003 mgm. and 0·004–0·0005 = 0·0035 mgm. of copper-head venom are neutralized by 0·2 c.c. of this antivenine. This antivenine neutralizes between ·015 and ·0175 mgm. per c.c.

PROTOCOL 6.—*Death-Adder Antivenine against Death-Adder Venom.*

(*a*) PROTECTION TESTS ON GUINEA-PIGS.

Weight of guinea-pig, gm.	Approximate number of C.L.D.'s.	Weight of venom in mgm.	Result.
251	8	·300	Died in less than 20 hours.
267	5·5	·225	,, ,, ,,
230	6	·207	Died in 64 hours.
259	5	·200	Moderate symptoms; survived.
235	5	·200	,, ,,
252	5	·187	Died in less than 48 hours.
247	5	·187	Died in less than 40 hours.
274	4·3	·175	No symptoms; survived.
269	4·3	·175	,, ,,
280	4	·168	Moderate symptoms; survived.
265	3·5	·150	No symptoms; survived.

The certainly lethal dose of death-adder venom is 0·015 mgm. per 100 gm. This antivenine neutralizes between 0·175–0·04 = ·135 and ·200–·035 = ·165 mgm. per c.c.

(*b*) TESTS ON THE ISOLATED PLAIN MUSCLE.

Right horn.			Left horn.		
Antivenine.	Venom.	Reaction.	Antivenine.	Venom.	Reaction.
0·2	0·1	Nearly maximal	...	0·006	Small.
0·2	0·09	½ maximal	...	0·01	⅔ maximal.
0·2	0·07	Small	...	0·005	Very small.
0·2	0·066	⅔ maximal	...	...	...
0·2	0·06	*Nil*	0·2 (death-adder)	0·07	Stepped, ½ maximal.
0·2	0·055	,,	...	0·004	½ maximal.
0·2	0·050	,,	...	...	...
...	...	...	0·2 (horse-serum)	0·005	⅔ maximal.
...	...	...	Do.	0·002	*Nil.*

The minimal effective dose is 0·005. Between 0·060–0·005 = 0·055 mgm. and 0·07–0·005 = 0·065 mgm. is neutralized by 0·2 c.c. of antivenine. The antivenine neutralizes between ·275 and ·325 mgm. per c.c.

PROTOCOL 7.—*Death-Adder Antivenine against Tiger-Snake Venom.*

(*a*) PROTECTION TESTS ON GUINEA-PIGS.

Weight of guinea-pig, gm.	Approximate number of C.L D.'s.	Weight of venom in mgm.	Result.
255	29·5	0·15	Died in less than 20 hours.
244	28·5	0·14	,, ,, ,,
239	27	0·13	,, ,, ,,
255	25	0·12	,, ,, ,,
282	20	0·11	,, ,, ,,
228	22	0·1	,, ,, ,,
235	19	0·09	,, ,, ,,
240	16·5	0·08	Died in 68 hours.
238	15	0·075	Moderate symptoms; survived.
235	15	0·07	Very severe symptoms; survived to 4th day.
234	15	0·075	Moderate symptoms; survived.
262	11·5	0·06	Died in 68 hours.
260	10	0·05	No symptoms; survived.
237	8·5	0·04	,, ,,
259	6·5	0·035	,, ,,
268	5	0·03	,, ,,

The certainly lethal dose of tiger-snake venom is 0·002 mgm. per 100 gm. This antivenine neutralizes between 0·05–0·005 = 0·045 and 0·08–·005 = 0·075 mgm. of tiger-snake venom per c.c.

(*b*) TESTS ON THE ISOLATED PLAIN MUSCLE.

Right horn.		Left horn.	
Antivenine.	Venom (tiger).	Reaction.	Venom.	Reaction.
0·2	0·15	Maximal	0·002	$\frac{2}{3}$ maximal.
0·2	0·05	,,	...	...
0·2	0·015	$\frac{2}{3}$ maximal	0·001	$\frac{1}{2}$ maximal.
0·2	0·014	,,	0·001	Nearly maximal.
0·2	0·013	*Nil*	0·001	,,
0·2	0·012	,,	...	...
0·2	0·01	,,	...	...
0·2	0·008	,,	0·008 (0·2 tiger antivenine)	Small.

The minimal effective dose (M.E.D.) of tiger-snake venom is 0·0005. Between 0·013–0·0005 = 0·0125 mgm. and 0·014–0·0005 = 0·0135 mgm. are neutralized by 0·2 c.c. of antivenine. Death-adder antivenine neutralizes between 0·0625 and 0·0675 mgm. per c.c.

DISCUSSION.

These results may be summarized as follows:

		Neutralization by 1 c.c. of antivenine.			
		Protection tests.		Tests with plain muscle.	
Antivenine.	Venom.	Approximate number of C.L.D.'s. for guinea-pigs weighing 250 gm.	Weight of venom in mgm.	Approximate M.E.D.'s. in bath of 30 c.c.	Weight of venom in mgm.
Tiger	Tiger	22	0·105–0·115	80	0·0375–0·0425.
,,	Copper head	2	0·024–0·035	44	0·022.
,,	Death adder	0·5	0·019	22	0·1 –0·125.
Death adder	,,	4	0·135–0·165	60	0·275 –0·325.
,,	Copper head	2	0·031–0·036	32	0·015 –0·0175.
,,	Tiger	12	0·045–0·075	130	0·0625–0·0675.

It is evident that save in the titration of tiger-snake antivenine against copper-head venom, the method of testing by the reaction of the isolated plain muscle gives no indication of the protective value of the two antivenines tested. In view of the complexity of these venoms this result is not unexpected. Even if the stimulant substances were identical with the neurotoxic principle (to which, following the subcutaneous injection of tiger-snake and copper-head venoms, the toxic effects are mainly due) anomalous results might be expected with death-adder venom and antivenine, for in the guinea-pig at least, the intense hæmorrhagic lesions in the lungs play a large part in causing the fatal issue. A further factor which might be expected to play a part in the result is the much lower potency of death-adder venom in stimulating the plain muscle. It is rather suprising in view of this fact that the titre of death-adder antivenine against the venoms of both the death adder and tiger snake should be so high as measured by the isolated plain muscle. Otherwise the results of the plain muscle titration, though quantitatively different from those obtained from protection experiments in the guinea-pig, support them in displaying a certain group specificity in the Australian venoms. Tidswell (1906) found that his tiger-snake antivenine (1·0 c.c. of which neutralized 1·25 mgm. of tiger-snake venom) was highly specific in its action, but the dosage which he used did not exclude the possibility of its exerting some protective action against less than 10 certainly lethal doses of the venoms tested—those of the black snake, brown snake and death adder. Martin (1905) found that Tidswell's tiger-snake antivenine was effective against the thrombase activity of the black snake (*Pseudechis porphyriacus*). Space forbids any extensive discussion of the literature on the specificity of antivenines which Phisalix (1922) has admirably reviewed. Though antivenines made with a single venom are only highly effective against that venom, there are numerous instances of partial protective effect against the venoms of closely allied snakes. Such appears to be the case with the two low-potency antivenines studied here, notably in regard to the protection afforded by death-adder antivenine against tiger-snake venom.

It might be argued that horse-serum itself would exert a protective effect of the order observed in these experiments. This is not the case, for guinea-pigs

which were injected subcutaneously with mixtures of 1 c.c. of horse-serum with 1, 1·25 and 1·5 C.L.D.'s of the venoms of the tiger snake, death adder and copper head (allowed to stand at room temperature for 1 hour before injection) all died in from 20 to 44 hours (except one animal of two which received one certainly lethal dose of death-adder venom, which recovered after a severe illness). That horse-serum has some slight protective effect is illustrated by the fact that of 2 guinea-pigs which received mixtures of 2·0 c.c. of horse-serum with 1 and 1·25 certainly lethal doses of copper-head venom both survived, though 2 which received similar mixtures with tiger-snake venom instead of copper-head died in 44 and 68 hours.

The other possibility that must be considered in discussing the genuineness of the non-specific effect displayed here is that of admixture of venoms used for immunization. The venoms have been personally collected by Dr. N. H. Fairley and myself, and the greatest care has been taken to avoid any contamination of one venom by others, the technique of collection and of handling the venoms when collected having been carefully standardized for this purpose. Further evidence that this possibility cannot account for the results is afforded in the case of the death-adder antivenine. This has a powerful protective effect against tiger-snake venom in neutralizing between 10 and 15 certainly lethal doses. The stock death-adder venom used for immunization has been repeatedly tested in the course of other experiments (Kellaway, 1929), using doses of the order of 40 mgm. for intravenous injection into rabbits. In no case has intravascular coagulation occurred. Since tiger-snake venom in minute dosage will produce this effect, it is certain that no admixture of tiger-snake with death-adder venom has occurred.

CONCLUSIONS.

1. All the Australian elapine venoms tested have a stimulant action upon isolated plain muscle.

2. The type of action resembles the anaphylactic reaction of the isolated plain muscle of the guinea-pig in its latent period, and in the ease with which the muscle is desensitized by the venoms of this group.

3. The Australian venoms produce the reaction in varying concentrations. After reaction to one venom the plain muscle is insensitive to equivalent doses of the other venoms in the group.

4. The muscle after desensitization does not recover its sensitiveness to snake venom, but its reactions to other stimuli are unimpaired.

5. The plain muscle of guinea-pigs sensitized by the injection of egg albumin, after desensitization with snake venom, retains unimpaired its sensitiveness to its specific anaphylactic antigen. After desensitization with egg albumin the muscle appears to be somewhat less sensitive to snake venom.

6. Experiments with ergotoxin suggest that the effect of the venoms is a direct one upon the plain muscle.

7. The venoms cause contraction of the isolated uterus of the rat, and the reaction of the plain muscle of the guinea-pig and rabbit is therefore not due to the production of histamine, since this drug causes relaxation, not contraction, in the uterus of the rat.

8. The reaction differs from that caused by the toxin of *Vibrion septique*, and after desensitization with snake venoms a good reaction can be obtained with this toxin.

9. The behaviour of the only two other venoms tested (those of *Naia tripudians* and *Vipera russelli*) is quantitatively widely different from those of the Australian elapines.

10. The stimulant principle in the Australian venoms is moderately thermolabile, and is destroyed at 92°–94° C. at about the same rate as the neurotoxic principle.

11. Like the neurotoxic principle, it appears to reside in the proteose fraction of the venom.

12. At pressures of 300 to 400 mm. of mercury, and during a period of some hours, it only passes with difficulty through pyroxylin filters permeable to erythro-dextrins but not to egg albumin or hæmoglobin.

13. The stimulant principle is neutralized by antivenine, but the results obtained by titration, using the isolated plain muscle, are quantitatively widely different from those obtained by subcutaneous injection in guinea-pigs.

14. Death-adder and tiger-snake antivenines give some protection against Australian venoms other than those against which they are prepared, and non-specificity of the same order is observed in tests with the isolated plain muscle.

I wish to acknowledge my indebtedness to Dr. N. Hamilton Fairley, who collected much of the Australian venom for me; to Dr. F. G. Morgan, Director of the Laboratories Division of the Commonwealth Department of Health, for the tiger and death-adder antivenines; to Col. T. P. Mackie, who gave me supplies of the venoms of the cobra and daboia; to Mr. Kinghorn and to Mr. Thomson, who provided supplies of the venom of *Oxyuranus maclennani;* and finally to Dr. Trevan, who kindly furnished me with the *Vibrion septique* toxin.

My thanks are also due to Mr. H. F. Holden, who has advised me on the chemical aspects of this problem; to Miss Mavis Freeman, who prepared for me the crystalline egg albumin used in these experiments, and with whose co-operation the experiments on fractionation were carried out; and finally to Miss Beryl Splatt, who weighed out for me numerous samples of venom.

REFERENCES.

Adair, G. S.—(1915) *Proc. Roy. Soc.* A, **108**, 627.
Buttle, G. A. H., and Trevan, J. W.—(1928) *Brit. J. Exp. Path.*, **9**, 182.
Dale, H. H.—(1913) *J. Pharm. Exp. Therap.*, **4**, 167.
Fairley, N. H.—(1929) *Med. J. Aust.*, **1**, 377.
Gunn, J. A., and Heathcote, R. StA.—(1921) *Proc. Roy. Soc.*, B, **92**, 81.
Kellaway, C. H.—(1928) *J. Path. Bact.*, **31**, 142; (1929) *Med. J. Aust.*, **1**, 348, 358, 764, 772.
Kinghorn, J. R.—(1923) *Rec. Aust. Mus.*, **14**, 42.
Martin, C. J.—(1893) *J. Physiol.*, **15**, 380; (1895) *J. Roy. Soc. New South Wales*, **29**, 146; (1896) *ibid.*, **30**, 147; (1905) *J. Physiol.*, **32**, 207.
Phisalix, M.—(1922) 'Animaux venimeux et venins,' **2**, 722 (Masson et Cie, Paris).
Stockhard and Papanicolaou.—(1919) *Biol. Bull. Woods Holl.*, **4**, 37.
Tidswell, F.—(1906) 'Researches on Australian Venoms,' Sydney, N.S.W. (Govt. Printer).

COMPARISON OF THE PATHOLOGICAL EFFECTS OF PROLONGED EXPOSURE TO CARBON MONOXIDE WITH THOSE PRODUCED BY VERY LOW OXYGEN PRESSURE.

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Received for publication July 25th, 1929.

A STUDY of the more recent reviews dealing with carbon monoxide (Sayers and Davenport, 1925; Bock, 1923) indicates that the physiological effects and symptoms of this poison are generally regarded as resembling closely those produced by severe anæmias and by breathing oxygen at low pressures in the air; in other words so far as man is concerned carbon monoxide is not a poison beyond interfering with the transportation of oxygen by hæmoglobin. J. S. Haldane's name in particular has been associated with this view. There is some evidence (Warburg, 1926; Haldane, 1927; Keilin, 1929) that carbon monoxide in relatively high proportions does act as a tissue poison, but it has yet to be proved that such is the case with the very small percentages which are sufficient to poison man.

In some physiological experiments (Campbell, 1927, 1928[1]) dealing chiefly with the effects of carbon monoxide upon tissue oxygen tensions I have observed a striking resemblance to the effects (*a*) of hæmolysis, (*b*) of bleeding, and (*c*) of breathing oxygen at very low pressure. In the same researches I have made a fairly complete histological examination of the tissues of animals exposed for prolonged periods to low oxygen pressure and to carbon monoxide, and record the pathological findings regarding carbon monoxide in the present paper. The literature concerning the pathology appears at a first glance to be extensive, but a good deal of it has been built up from post-mortem examinations of human beings, some of these examinations being delayed for some days after death; also in some cases, which seem to have obtained undue prominence, the pathological data described were those due to secondary effects, *e.g.* pernicious anæmia, following acute poisoning with illuminating gas. Sayers and Davenport (1925) summarize their opinion of the previous literature as follows: "A knowledge of the pathology of carbon monoxide poisoning has developed rather slowly, due to unscientific methods of conducting investigations, and to lack of knowledge of the processes of the human body on the part of the investigators. The most obvious pathological effects of the poison were noted by physicians in connection with the symptoms observed in the victims of this gas, but even to-day, with all the advance that has been made, investigators are not in absolute accord as to just what takes place in the body in the presence of quantities of carbon monoxide inimical to life." It is not considered that my present results fulfil all the above requirements, but since

they do show the striking resemblance between the pathological effects of prolonged exposure to carbon monoxide—that is, of chronic carbon monoxide poisoning—and those due to prolonged exposure to low oxygen pressure in the air, it is considered that acute poisoning by carbon monoxide would resemble just as closely the acute derangement due to sudden exposure to very low oxygen pressure in the air if the conditions regarding suddenness, duration and degree were made identical in the two cases. At the same time, it must be remembered that illuminating gas contains other harmful gases besides carbon monoxide; we are concerned here chiefly with pure carbon monoxide.

PLAN OF THE INVESTIGATION.

The animals—5 rabbits, 6 guinea-pigs, 6 rats, 12 mice—were enclosed in respiratory chambers which have been described in detail elsewhere (Campbell, 1927, 1928[1]). As a rule the chambers were opened for only about ten to twenty minutes once every third or fourth day, to replace food, bedding and water, so that the animals were breathing carbon monoxide almost continuously for the periods specified in Tables I, II and III. Details *re* food, etc., will be obtained from the papers mentioned above.

TABLE I.—*Attempted Acclimatization of Rabbits Nos. 50, 51, 52 and 53 to Carbon Monoxide.*

Time (days).	CO in inspired air % atmosphere.	Weight of rabbits in grammes.				Deaths.
		No. 50.	No. 51.	No. 52.	No. 53.	
0	·100	2000	2300	2000	2700	..
7	·125	..	..	..	..	..
15	·130	1780	2430	1950	2820	No 50
23	·200	..	..	..	..	No. 51.
30	·200	..	2000*	1900	2820	..
43	·200	..	..	1860	2650	..
46	·250	..	..	1900	2650	..
49	·300	..	..	..	..	..
51	·300	..	..	1800*	2850*	Nos. 52 and 53 killed for histology.

* These animals had fluid in the body-cavities.

The percentage of carbon monoxide in the air within the chambers was estimated by means of the bottle or flask method, in which some of the air was shaken vigorously with diluted blood, the percentage saturation of this being determined by means of Hartridge's reversion spectroscope.

The required amount of pure carbon monoxide—prepared from formic and sulphuric acids—was added carefully to the air in the chamber. The carbon dioxide produced by the animal was absorbed by soda-lime, and the oxygen used up was automatically replaced by oxygen bubbling through a water valve from a spirometer.

TABLE II.—*Attempted Acclimatization of 6 Guinea-pigs, 6 Rats and 12 Mice to Carbon Monoxide.*

Time (days).	CO in inspired air % atmosphere.	Average weight in grammes. Guinea-pigs.	Rats.	Mice.	Deaths.
0	·070	560	76	21·5	..
15	·110	..	..	..	G.P. No. 1.
18	·110	492	71	18·6	Rats Nos. 1 and 2.
19	·130	..	..	..	..
22	·200	..	..	..	..
25	·200	..	..	..	Rat No. 3.
27	·250	..	..	..	G.P. No. 2.
28	·250	..	68	..	Mouse No. 1, Rat No. 4. Rats Nos. 5 and 6 killed.
32	·250	427	..	17·4	Mouse No. 2.
34	·250	..	..	..	G.P. Nos. 3, 4, 5 and 6.
36	·250	..	..	17·5	Mice Nos. 3 and 4.
46	·250	..	..	..	..
54	·250	..	..	16·8	Mice Nos. 7, 8 and 9.

TABLE III.—*Attempted Acclimatization to Carbon Monoxide: Rabbit No. 54.*

Time (days).	CO in inspired air % atmosphere.	Hb not saturated with CO % of normal.	O_2 tensions in tissues mm. Hg. Skin.	Abdominal cavity.	Weight (kgm.).	Hb %.	Red cells per c.mm. millions.
0	0	100	19	32	2·16	64	5·0
3	·065	74	16	26	..	..	..
7	·080	70	15	23	..	..	..
10	·120	63	15	23	..	..	..
14	·120	60	14	23	..	80	7·5
17	·140	55	14	23	2·12	..	..
21	·140	57	14	21	..	..	..
24	·165	50	14	21	..	..	..
28	·160	52	14	21	..	95	8·5
31	·170	49	13	20	..	..	..
35	·225	43	13	19	..	..	..
37	·240	43	10	16	1·85	100	9·0

By exposing the animals at first to a relatively low percentage of carbon monoxide and then gradually increasing the concentration by stages a considerable degree of toleration was established. Thus animals exposed immediately to 0·150% of carbon monoxide died in three days, but some of those used in the experiments illustrated in Tables I, II and III tolerated about 0·250% for several days at least. Some of the animals were found dead: the others—2 rats, 2 rabbits, 5 mice—were killed so that their tissues were quite fresh for histological examination. The histological evidence obtained from the animals

which died in every respect confirmed that obtained from the animals which were killed.

EFFECTS OF PROLONGED EXPOSURE TO CARBON MONOXIDE.

The individual differences between powers of resistance, of animals of the same kind, to carbon monoxide which have been observed by previous researchers were evident in the present observations. As regards different classes of animals, I found rabbits and mice rather more resistant than rats and guinea-pigs; I obtained similar evidence for rabbits as regards resistance to prolonged exposure to low oxygen pressure in the inspired air, and also in these low oxygen experiments mice and rats were more resistant than guinea-pigs, cats and a monkey. Bock (1923) records some evidence that rabbits and guinea-pigs were more resistant to carbon monoxide than mice and rats, but his conditions were rather different to mine.

One of the objects of the present research was to endeavour to determine what rendered the animals resistant. From Nasmith and Graham's (1906) earlier work it was obvious that under gradual increase in percentage of carbon monoxide breathed, an increase of red cells per cubic millimetre of blood enabled guinea-pigs to tolerate for some time much higher percentages of the poison than when suddenly exposed without any previous acclimatization. I found similar increases in red cells in all my rabbits. Nasmith and Graham's guinea-pigs were able to maintain body-weight so long as their blood was not saturated more than 25% with carbon monoxide, the increase in red cells then being capable of making good the loss in oxygen capacity of the blood. This saturation indicates only a very low percentage of carbon monoxide in the inspired air.

My animals underwent a rather more severe test, but were given time to acclimatize fairly slowly to gradually increasing percentage of carbon monoxide, and even when the red cells and volume of air breathed were markedly increased the oxygen tensions in the tissues were much below normal; at the same time the animals lost appetite and weight, some of them dying fairly early in the experiment and others surviving the highest percentage of poison tested. I found a markedly subnormal oxygen tension in the tissues even in an animal which was very resistant (Table III). From this it seemed probable that, just as when breathing oxygen at very low pressure in the air, the power to tolerate carbon monoxide depended upon power to tolerate a low oxygen tension in the tissues, and not upon the power of the animal to keep the tissue oxygen tension at normal level, because no animal tested was able to do this. The hypothesis put forward is that if the heart muscle were able to tolerate a low oxygen tension and to keep the circulation going well the rest of the tissues would be able to function well enough, but if the heart muscle failed to tolerate a surrounding low oxygen tension, then circulatory failure would set in and lower the oxygen tension in the tissues still further. Thus in the animals that failed if this theory was correct evidence of dilatation of the heart with the usual signs of circulatory failure—congestion, œdema, dropsy, degeneration, atrophy—should be obtained. This is just what did occur; in all the animals there was evidence of capillary and venous engorgement, the animals which

died early showing just as marked changes in their tissues as the animals which died some weeks later; the degree of damage done was therefore independent of length of exposure to the poison, and the earlier demise of the less resistant animals was due to failure of the heart occurring sooner than in the more resistant animals. It is not suggested that other organs besides the heart were not directly affected by the low oxygen tension in the tissues; no doubt they were, but the level to which the oxygen tension in the other tissues fell depended upon the powers of the heart to function under a low tissue oxygen tension.

It is well known that in heart failure the main feature is loss of tone of the muscle with dilatation, and there may be little or no histological change in the heart-muscle itself; evidence of heart failure is conclusive if the other tissues reveal damage due to venous congestion. In many of my animals the heart-muscle near the epicardium exhibited atrophy due to excessive venous engorgement; a few—*e. g.* rabbit, guinea-pig—showed marked fatty degeneration; why this degeneration should appear in such a marked degree in some animals and not be present at all in others of the same class is a mystery. In many cases the heart-muscle striations were poorly represented, but in some there was no obvious histological change. The most striking change observed was the venous engorgement, particularly near the epicardium. The congestion was often so great that the vascular bed occupied a greater area of the field than the heart-muscle fibres, which exhibited atrophic changes. This resembled very closely the condition illustrated in a previous research, showing the effects of breathing air with oxygen at very low pressure for long periods (Campbell, 1928[2]); indeed this previous illustration would do equally well for carbon monoxide poisoning.

As might be expected, the organs which showed the greatest congestive changes were the liver and the lungs; the brain also often revealed marked congestion. The other organs—spleen, kidney, suprarenal, thyroid—sometimes exhibited similar effects. Although in chronic carbon monoxide poisoning the red cells per cubic millimetre of blood are greatly increased and the blood becomes more viscous, this was not the cause of the stasis, because a rabbit with red cells increased to 80 % above normal did not show an exceptionally high degree of congestive change.

The liver was the organ whose cells showed greatest change, these being often greatly atrophied and appearing like threads of a network, the spaces of which were filled with blood; here again the effect of chronic carbon monoxide poisoning was exactly the same as that of breathing oxygen at very low pressure in the air for long periods. An illustration given for the latter condition in a previous paper (1928[2]) would do just as well for the former condition. Further, a few animals exhibited fatty degeneration of the liver.

The lungs sometimes exhibited cells with pigment-granules characteristic of chronic venous congestion; the capillaries were often excessively congested and there was sometimes œdema and patches of emphysema.

Table IV gives full details of the congestive changes in the organs chiefly affected; obvious congestion is represented by the sign +, and marked congestion with its accompanying changes by the sign ++. There was little doubt that failure to tolerate prolonged exposure to carbon monoxide depended upon

TABLE IV.—*Congestion of Organs due to Carbon Monoxide.*

Animal.	Exposure to CO (days).	Highest % of CO in air; % atmosphere.	Died (D) or killed (K).	Congestion of organs (obvious, +, marked, + +).			
				Liver.	Lung.	Heart.	Brain.
Rabbit No. 50	15	·130	D	++	++	+	+
„ 51	23	·200	D	++	++	++	++
„ 52	51	·300	K	+	++	++	+
„ 53	51	·300	K	+	+	++	+
„ 54	37	·240	K	+	+	+	+
G.P. No. 1	15	·110	D	++	++	+	++
„ 2	27	·250	D	++	++	++	++
„ 3	34	·250	D	++	++	++	++
„ 4	34	·250	D	++	++	+	++
„ 5	34	·250	D	++	++	+	++
„ 6	34	·250	D	++	++	+	++
Rat No. 1	18	·110	D	++	++	++	..
„ 2	18	·110	D	++	++	++	..
„ 3	25	·200	D	++	++	++	..
„ 4	28	·250	D	++	++	++	++
„ 5	28	·250	K	++	++	++	++
„ 6	28	·250	K	++	+	++	+
Mouse No. 1	28	·250	D	++	++	++	..
„ 2	32	·250	D	++	+	++	..
„ 3	36	·250	D	++	+	++	..
„ 4	36	·250	D	++	+	++	..
„ 5	54	·250	K	++	++	++	+
„ 6	54	·250	K	++	++	++	+
„ 7	54	·250	D	++	++	++	..
„ 8	54	·250	D	++	++	++	..
„ 9	54	·250	D	++	++	++	..
„ 10	54	·250	K	+	+	++	+
„ 11	54	·250	K	+	+	++	+
„ 12	54	·250	K	++	++	++	+

the heart; we are dealing here with chronic poisoning. In cases of carbon monoxide poisoning in man the conditions are usually different; that is, the person has been exposed very suddenly to acute poisoning, and in such a case the blood becomes very highly saturated with the poison, so that the tissue oxygen tensions must fall to a very low level, judging from the effects I have observed in rabbits. A sudden exposure to 0·15 % of carbon monoxide in the air lowered the oxygen tension in the abdominal cavity from the normal figure of about 35–40 mm. Hg. to the dangerous level of 16–20 mm. Hg.; at the same time the oxygen tension in the outlying tissues, such as under the skin, was reduced to 1–9 mm. Hg. in place of the normal figures of about 20–25 mm. Hg. Under such low oxygen tensions in the tissues, the cell's structure probably disintegrates and poisonous by-products result, so that even if a person is removed from the poisonous air before he is dead, whether he recovers or

not depends entirely upon the damage done to the tissues by the extreme asphyxiation. This theory is not new, but the evidence from my results for oxygen tension in the tissues indicated clearly to what a very low level the oxygen tension in some of the tissues fell without death following immediately. Sudden death probably occurs because the heart-muscle fails to withstand the very low oxygen tension. Probably undetected changes in the brain-cells occur varying in degree with the level to which the tissue oxygen tension falls, and these changes explain the numerous psychical troubles which Mott (1907) has recorded. He did find hyperæmia of the brain with numerous capillary hæmorrhages in his acute cases of poisoning with illuminating gas. In similar cases Gürich (1925) found areas of degeneration with evidence of bleeding in the heart muscle. In the chronic poisoning of my animals with carbon monoxide congestion of the brain was noted, but there did not appear to be

TABLE V.—*Congestion of Organs due to Low Oxygen Pressure.*

Animal.	Exposure to low O_2 (days).	Lowest % of O_2 in air.	Died (D) or killed (K).	Congestion of organs (obvious, +; marked, ++).			
				Liver.	Lung.	Heart.	Brain.
Rabbit No. 11	33	7·1	D	++	++	++	..
G.P. No. 1	24	8·8	D	++	+	++	+
,, 2	24	9·5	K	++	++	++	+
,, 3	19	9·5	D	++	++	++	..
Rat No. 1	15	11·4	K	++	++	+	..
,, 2	15	11·4	K	+	++	+	..
,, 3	15	11·4	K	++	++	++	..
Mouse No. 1	45	10·0	D	++	+	+	++
,, 2	45	10·0	K	++	+	+	++
,, 3	45	10·0	K	++	+	+	++
,, 4	45	10·0	K	++	+	+	+
,, 5	53	7·2	K	++	++	++	..
,, 6	53	7·2	D	++	++	++	..

any striking histological change in the cells apart from some minor evidence of chromatolysis. Mott stated that he had not seen the same picture, *i. e.* of numerous hæmorrhages, in any other condition, and thought that carbon monoxide had some special action apart from depriving the tissues of oxygen. The true explanation of this may be that the tissues had not been so acutely asphyxiated in the other conditions studied. I found (1927, 1928[1]) that bleeding, hæmolysis, etc., did not reduce the oxygen tension in the tissues to the same low level as carbon monoxide for a similar loss of function of hæmoglobin, and consider that if it were possible to imitate exactly the asphyxiation produced by carbon monoxide, very similar effects could be produced by low oxygen pressure in the air. This statement is made because the pathological effects I have observed for chronic carbon monoxide poisoning resemble very closely those observed after prolonged exposure to very low oxygen pressure in the air. In Table V, I have collected together pathological results

from a few of my numerous animals which were exposed in previous experiments for prolonged periods to gradually lowered oxygen pressure in the inspired air. Comparison of these results with the data in Table IV reveals the striking resemblance between the effects of the two conditions. Any histological slide obtained in the experiments with carbon monoxide could be matched very closely with one obtained from the experiments in which oxygen was breathed at very low pressure. This is in accordance with Haldane's view that carbon monoxide acts merely by depriving the tissues of oxygen.

SUMMARY.

It is shown that in chronic carbon monoxide poisoning in animals the pathological effects resemble very closely those of prolonged exposure to very low oxygen pressure in the inspired air; the main changes are venous congestion with atrophy of certain cells (*e. g.* liver, heart muscle near epicardium), œdema and dropsy indicating that heart failure is the cause of inability to tolerate prolonged exposure to carbon monoxide. The organs showing most marked congestion and its sequelæ are the liver, lungs, heart muscle near epicardium and the brain. Haldane's view that carbon monoxide acts purely by interfering with the oxygen supply to the tissues is supported.

Power to tolerate carbon monoxide—just as in the case of low oxygen pressure in the air—depends upon the ability of the vital organs, particularly the heart, to continue to function under a low tissue oxygen tension; tissue oxygen tension is not brought back to normal level by so-called acclimatization under severe degrees of oxygen want.

I am indebted to Dr. P. P. Laidlaw, F.R.S., and to Dr. Perdrau for advice regarding the pathological changes.

REFERENCES.

Bock, J.—(1923) 'Handbuch der experimentelle Pharmakologie.'
Campbell, J. A.—(1927) *J. Physiol.*, **62**, 211; **63**, 325.—(1928[1]) *Ibid.*, **65**, 255.—(1928[2]) *Lancet*, **2**, 84.—(1929) *J. Physiol.* (in the press).
Gürich.—(1925) *München. med. Wschr.*, **72**, 2194.
Haldane, J. B. S.—(1927) *Biochem. J.*, **22**, 1068.
Keilin, D.—(1929) *Proc. Roy. Soc.*, B, **104**, 223.
Mott, F. W.—(1907) *Arch. Neurol.*, **3**, 246.
Nasmith, G. G., and Graham, D. A. L.—(1906) *J. Physiol.*, **35**, 32.
Sayers, R. R., and Davenport, Sara J.—(1925) *Publ. Health. Bull.*, No. 150, U.S. Publ. Health Service.
Warburg, O.—(1926) *Biochem. Z.*, **177**, 471.

THE OCCURRENCE OF MITOSES IN NORMAL AND MALIGNANT TISSUES *IN VITRO*.

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Forwarded for publication July 29th, 1929.

THE very moderate growth-rate characteristic of malignant tissues cultivated *in vitro* and the much more abundant proliferation of normal tissues cultivated under equally favourable conditions have for a long time presented a very considerable problem to students of cell biology. When one studies a culture of cancerous tissue which has been fixed and prepared for histological examination, one is immediately struck by the unusual number of mitotic figures which are to be seen—a number which far exceeds that observed in corresponding areas of normal tissues. In seeking an explanation for such findings, one is immediately led to question the supposed relationship between cell division and tissue growth in the sense of an increase in mass. Strangeways (1924) has already indicated that tissue growth may be only an incidental result, not the primary function of cell division.

Such observations as these suggested a comparative study of the frequency of cell division in normal and malignant tissues cultivated *in vitro*. At the same time it was hoped that we might determine with some degree of accuracy the region of greatest proliferation within a culture. Since we must consider a tissue culture as an independent organism in many respects, we should make some effort to study its anatomy. The investigation which has been undertaken has a much larger scope than will be discussed at this time. Consequently, of the material which has been studied, reference will be made here to but one representative culture from each of the two types of tissue with which we are concerned in the present report—the one type being normal, the other malignant.

MATERIALS AND METHODS.

Culture No. 11962(F), the first to be dealt with, came from a strain of so-called fibroblasts in the eighth passage, a strain which was isolated from the zone of outgrowth from periosteum and bone removed from a 16-day-old chick embryo. This strain had been very early freed from all traces of the original explant. The culture medium which was employed was one most favourable to proliferation, and consisted of equal parts of chicken plasma and chick embryo juice. The culture was 24 hours old at the time of fixation. Helly's mixture was employed as fixative. Serial sections were prepared and

* Working under a joint fellowship from the National Research Council, Washington, and the Rockefeller Foundation, New York.

stained in hæmatoxylin (Mink) and eosin. This culture will hereafter be referred to as the *normal fibroblast culture.*

The second, 13296(M), is a 48-hour-old cover-glass culture of Ehrlich adeno-mouse-carcinoma (Frankfurt strain; in the 250th passage), which had been cultivated according to the usual method in a medium which consisted of equal parts of chick embryo juice and a plasma mixture of 25% chicken plasma

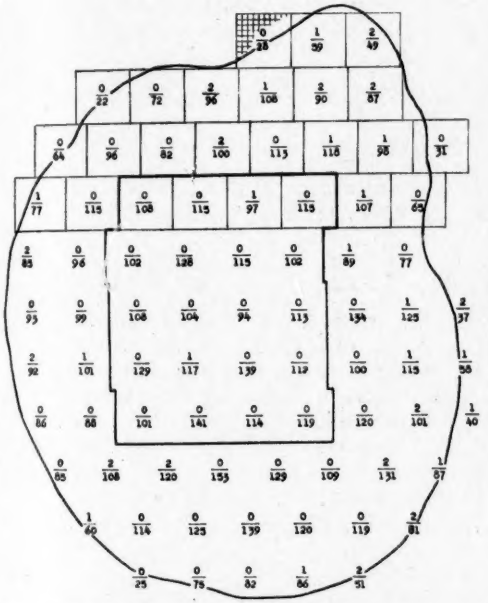

FIG. 1.—Normal fibroblast culture, 11962(F). Topographical diagram of the 7th section. Numerical values ($\frac{m}{c}$) represent ratio of number of mitoses (m) to number of cells (c) for each consecutive area covered by ocular micrometer. Areas which make up "central zone" indicated by enclosure. Compare Table II. (Figs. 1, 2, 5 and 6 were drawn to the same scale.)

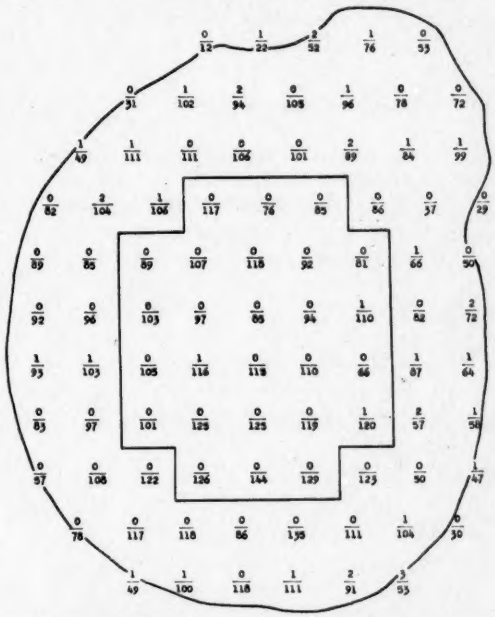

FIG. 2.—Normal fibroblast culture, 11962(F). Topographical diagram of the 8th section. Compare Table III.

and 75% rat plasma. The culture was fixed in Susa's fixative, cut in serial sections and stained with hæmatoxylin and eosin. It will subsequently be referred to as the *mouse carcinoma culture.*

The cultures, thus prepared, were studied section by section under a binocular microscope. The total number of cells occurring in each section was computed as accurately as was possible by counting the nuclei. At the same time note was made of the number of cells in the process of division. More specifically, the method employed was as follows: An ocular net micrometer (10 mm.^{2}) was inserted in one of the oculars. After the cells and accompanying

cell divisions occurring in a definite area of the section under study had been counted, the preparation was moved by means of the mechanical stage bringing the adjoining area into the field of the ocular micrometer. This procedure was repeated systematically until the whole section had been covered. At the same time the number of cells counted in each field, the number of these in division and the position of the margin of the culture (in the case of marginal areas) were recorded within the squared areas of a sheet of millimetre paper for each of the consecutive fields. Later, by cutting apart the rows in their order, and by matching the marginal outlines, the complete section could be reconstructed topographically, as is represented in Figs. 1, 2, 5 and 6. In these diagrams the positions of the numerical values $\frac{m}{c}$ (m = mitoses, c = cells) denote the centres of the squared fields. This simple procedure not only allowed one to ascertain the percentage of dividing cells for each section, and eventually for the whole culture, but it also furnished a series of maps from which comparisons of the different regions of the culture could readily be made.

THE NORMAL FIBROBLAST CULTURE.

This culture was selected by reason of its rapid rate of proliferation, as evidenced by the size which it had attained in the 24 hours following transplantation into new medium, and by reason of its very healthy condition. The tissue showed the utmost regularity in cell structure and absolute freedom from necrosis, as is exemplified by the photographic reproduction of the eighth section, which is presented here as Fig. 3. The seven sections which were studied yielded a total count of 32,447 cells, of which 258, or 0·8%, were recognized to be in mitosis at the moment of fixation (Table I). The remaining sections of the culture were not studied, due to the frequent inclusion of the plasma clot.

TABLE I.—*Normal Fibroblast Culture No. 11962(F).*

Section No.	Mitoses.	Cells.	$\frac{m}{c} \cdot 100$.
2	16	1256	1·3
3	25	2222	1·1
4	33	3577	0·9
5	47	4186	1·1
6	52	5697	0·9
7	45	7685	0·6
8	40	7824	0·5
2–8	258	32,447	0·8

Reference has already been drawn to the possibility of utilizing the data obtained in this study to determine the regions of greatest proliferation within the culture. From the topographical maps of the individual sections, which were furnished by the method employed, it has been possible to mark off roughly the central area of each section from the marginal area, and thereby

to determine the relative frequencies of mitosis occurring in each of these two zones. Figs. 1 and 2 show the zones which have been marked out for sections 7 and 8 respectively. The irregularities in outline resulted from the necessity of either including or excluding whole squares within the area of the zones. Tables II and III give the total number of cells occurring in the zones thus defined for these two sections, together with the percentage of those cells which were found to be in division. It is at once to be seen that the greatest

FIG. 3.—Normal fibroblast culture, 11962(F). Section No. 8. Helly; hæmatoxylin and eosin. Magnification, 1 : 58. Compare fig. 2.

frequency of mitosis for this culture occurs in the marginal areas. (The considerable variation which exists in the degree of difference between the outer and inner regions has no very great significance when one takes into account the artificial method which has been employed in their determination.) This is as we would expect. Normal tissue-cells, in order to remain healthy, require an adequate oxygen supply, just as they must have some means of escape from toxic concentrations of metabolic waste products. It is therefore not remarkable that the cells making up the marginal areas of the culture

should show a greater rate of proliferation than those which are forced to remain behind where conditions for life are no longer ideal.

TABLE II.—*Normal Fibroblast Culture No. 11962(F).*

Section No. 7.	Mitoses.	Cells.	$\frac{m}{c} \cdot 100$.
Marginal zone	43	5412	0·79
Central zone	2	2273	0·09
Total	45	7685	0·58

FIG. 4.—Mouse carcinoma culture, 13296(M). Section No. 27. Susa; hæmatoxylin and eosin. Magnification, 1 : 58. Compare fig. 6.

TABLE III.—*Normal Fibroblast Culture No. 11962(F).*

Section No. 8.	Mitoses.	Cells.	$\frac{m}{c} \cdot 100$.
Marginal zone	37	5069	0·73
Central zone	3	2755	0·11
Total	40	7824	0·51

THE MOUSE CARCINOMA CULTURE.

This culture was studied in its entirety with the exception of section 20 (mutilated in preparation) and a few sections at either extremity which contained too few cells to be of value. The 36 sections which were counted yielded a total of 70,028 cells, of which 4467, or 6·4%, were observed to be in

mitosis (Table IV). It will be seen that this percentage far exceeds that obtained for the normal culture (0·8%).*

TABLE IV.—*Mouse Carcinoma Culture No. 13296(M).*

Section No.	Mitoses.	Cells.	$\frac{m}{c} \cdot 100$.
3	1	46	2·2
4	2	104	1·9
5	5	163	3·1
6	17	357	4·8
7	26	467	5·6
8	24	584	4·1
9	26	630	4·1
10	17	549	3·1
11	22	712	3·1
12	17	856	2·0
13	50	1082	4·6
14	69	1300	5·3
15	73	1902	3·8
16	76	2301	3·3
17	119	3500	3·4
18	161	3734	4·3
19	213	3179	6·7
21	247	3551	6·9
22	194	3261	5·9
23	248	3454	7·2
24	238	3399	7·0
25	375	3993	9·4
26	321	4134	7·8
27	228	3803	6·0
28	267	3998	6·7
29	225	3228	7·0
30	199	3236	6·1
31	230	3150	7·3
32	236	2634	8·9
33	143	1924	7·4
34	152	1471	10·3
35	82	927	8·8
36	37	605	6·1
37	49	621	7·9
38	36	595	6·0
39	42	578	7·2
3–39	4467	70,028	6·4

* The objection may be raised that we are comparing avian tissue with mammalian tissue. In this connection, however, genetic relationships present no difficulties. We are dealing with a phenomenon common to all living matter, namely, proliferation, and not with any special properties of particular tissues.

Figs. 5 and 6 present diagrammatically the topography of sections 25 and 27 respectively. As before, the central areas have been marked off from the marginal areas, and the percentage of mitosis occurring in these two empirical zones has been determined for each section and presented in Tables V and VI. While there is a slightly greater frequency of mitosis to be found in the marginal areas of this culture, the excess is by no means as great as was demonstrated for the normal culture, and might very well lie within the limit of error. Section No. 27 is photographically reproduced in Fig. 4. While the picture is of little value cytologically due to the low magnification, it does, however, supplement the diagram given in Fig. 6, inasmuch as it shows the condition of the areas in which very few or no cells were registered. In some cases such areas were necrotic; in other cases they included widely dispersed

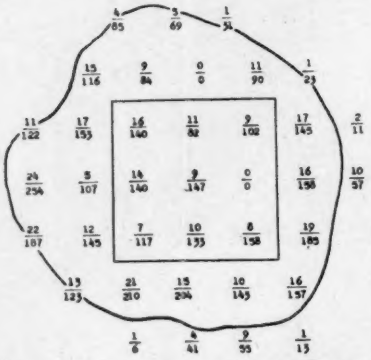

Fig. 5.—Mouse carcinoma culture, 13296(M). Topographical diagram of the 25th section. Compare Table V.

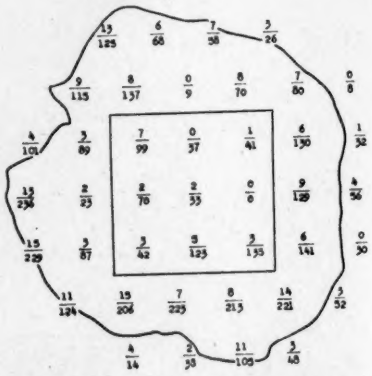

Fig. 6.—Mouse carcinoma culture, 13296(M). Topographical diagram of the 27th section. Compare Table VI.

heart-muscle cells (together with which the carcinoma was cultivated), which are easily distinguishable from the closely packed and more deeply stained infiltration of carcinoma cells, but which were not included in the count. Fig. 7 shows an area taken from the same culture in which seven mitoses may be seen lying very closely together—a condition which it would be most difficult to find in a culture of normal tissue.

Throughout the whole culture was to be found the most marked evidence of degenerative phenomena, ranging from cells with slightly abnormal nuclei, to areas containing mere shadowy remnants of pre-existing cell aggregates long since disintegrated. Nor were such degenerative changes restricted to cells which were at the moment of fixation in the resting condition. The area shown in Fig. 8 includes a cell which had met sudden death during the very process of cell division. Only the chromosomes are discernible, distinctly outlined against a field which is made up of the powdered remains of cells which had met the same fate. The cell body has disappeared. It was also

not unusual to find the chromosomes lying scattered over a very considerable area, as if the mother-cell had suffered a very violent disruption. But lying

FIG. 7.

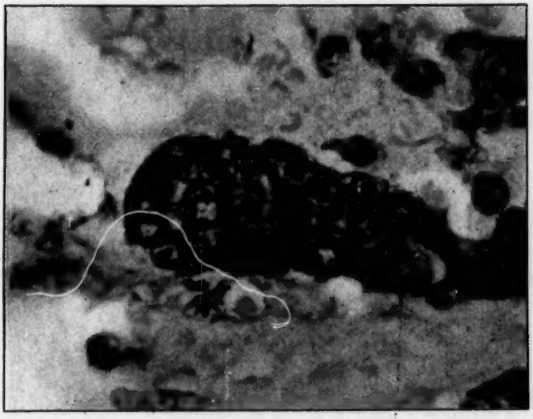

FIG. 8.

side by side with such disintegrating cells, and often in the very centre of extensive areas of necrosis, were to be found cell-nests whose individuals gave every appearance of health.

TABLE V.—*Mouse Carcinoma Culture No. 13296(M).*

Section No. 25.	Mitoses.	Cells.	$\frac{m}{c}$. 100.
Marginal zone .	291 .	2974 .	9·78
Central zone .	84 .	1019 .	8·24
Total . .	375 .	3993 .	9·39

TABLE VI.—*Mouse Carcinoma Culture No. 13296(M).*

Section No. 27.	Mitoses.	Cells.	$\frac{m}{c}$. 100.
Marginal zone .	205 .	3223 .	6·36
Central zone .	23 .	580 .	3·96
Total . .	228 .	3803 .	5·99

DISCUSSION.

It has been seen that we have in the case of the malignant tissue here referred to a very great number of dividing cells at any one moment—a number which far exceeds that found in normal tissues. We have always been accustomed to consider this strain of malignant tissue as one with a very limited capacity for growth *in vitro*. As an index for growth we have been forced to depend upon the increase in surface area, which we measure from day to day with the aid of projectoscope and planimeter. We are very well aware of the limitations of this method and of the very great error which it entails, for we have no measure of the thickness of the culture or of the density of the cells which populate it. However, in spite of these technical difficulties, we feel that we can categorically state that the carcinomatous tissue of this strain increases in mass at a rate which is appreciably less than that of normal tissues, even when such a statement must be based upon mere personal experience.

If, then, the tissue does not increase in mass at a rate which would be expected from the number of mitoses to be found therein at any particular moment, it may be possible that the process of cell division in the case of the carcinoma is of longer duration than it is in the case of normal tissue-cells, and thus at any one instant more cells are to be found in mitosis. But this supposition can be immediately disregarded. Cinematographic records of the process of cell division in these tissues have indicated that it is of no longer duration in the mouse carcinoma than in normal tissues.

Again, it might be suggested that the normal tissue-cells have some means of proliferation which does not involve mitosis and all of the accompanying changes by which we are accustomed to recognize it in living and fixed preparations. We believe, however, that we are justified in disregarding this possibility as well, in spite of the discussion which still centres around the question of amitosis and its occurrence in normal tissues.

This elimination brings us to the final possibility, which we believe must account, not only for the unusual frequency of cell division which we have found in malignant tissue, but also for the very limited increase in mass which accompanies it. The average duration of life of the carcinoma cell must be extremely short. A large percentage of the cells are dying at every moment. These losses are compensated for by the generation of new cells. When the generation of new cells exceeds the loss through death, growth results. Growth in a carcinoma culture is therefore not a necessary result of cell division, but is rather an index of the potencies of the component cells to initiate cell regeneration without the intervention of external stimuli.

It does not seem necessary to postulate that the mechanism which is responsible for this very unusual frequency of cell division, or cell regeneration, within the carcinoma culture, must be the same which allows of a rate of cell mitosis in the interior of the culture very nearly equal to that found in the exterior regions. And any explanation which is brought forward to account for these conditions must ultimately explain not only the origin of neoplastic growth in the organism, but also the *rôle* which it comes to play in the metabolism of the invaded area. It has been suggested in a previous study (Fischer, Laser and Meyer, 1929) that carcinoma-cells may be in a position to live and multiply over a considerable period on the expense of substances which arise through a breaking down of sister-cells. The observations which have made up the present report lead us to emphasize again this possibility.

SUMMARY.

1. The percentage of cells in mitosis was determined for fixed cultures of normal (fibroblasts) and malignant (carcinoma) tissue which had been cut in serial sections.

2. The percentage of cells in mitosis in malignant tissue was found to greatly exceed the corresponding percentage in normal tissue, in spite of the fact that the growth-rate of normal tissue may exceed that of malignant tissue by several hundred per cent.

3. In cultures of normal tissue the greatest number of dividing cells are to be found in the marginal areas.

4. In cultures of malignant tissue there is no appreciable difference between the number of dividing cells to be found in central and marginal regions.

5. The degenerative phenomena occurring in cultures of malignant tissue have been described and suggestions made as to their significance.

REFERENCES.

Fischer, A., Laser, H., and Meyer, H.—(1929) *Z. Krebsforsch.*, **29**, 270.

Strangeways, T. S. P.—(1924) 'Tissue Culture in Relation to Growth and Differentiation,' Cambridge.

A FILTRABLE FIBRO-SARCOMA OF THE FOWL.

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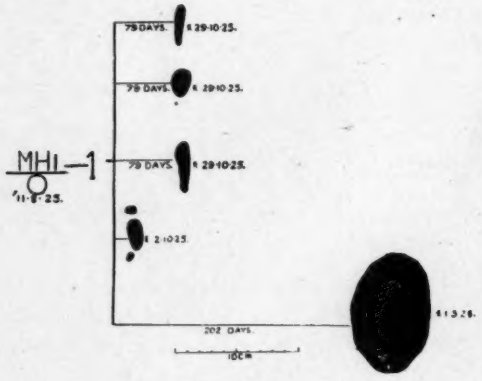
Received for publication August 18th, 1929.

A PRELIMINARY account of the early propagation of this tumour has already been published in this Journal (Begg, 1927). The primary growth (Plate—fig. 1) and first transplanted tumours have the structure of a simple hard fibroma. On account of a tendency of the tumours of the earlier series to show invasive powers and the occurrence in one bird of a lung metastasis, the tumour was described as a fibro-sarcoma.



TEXT-FIG. 1.

The primary growth was discovered at post-mortem on a white Leghorn hen. It was situated around a loop of small intestine and was uniformly hard throughout. Five white Leghorn chicks were inoculated with grafts in the right pectoral region. The grafts grew in all five.

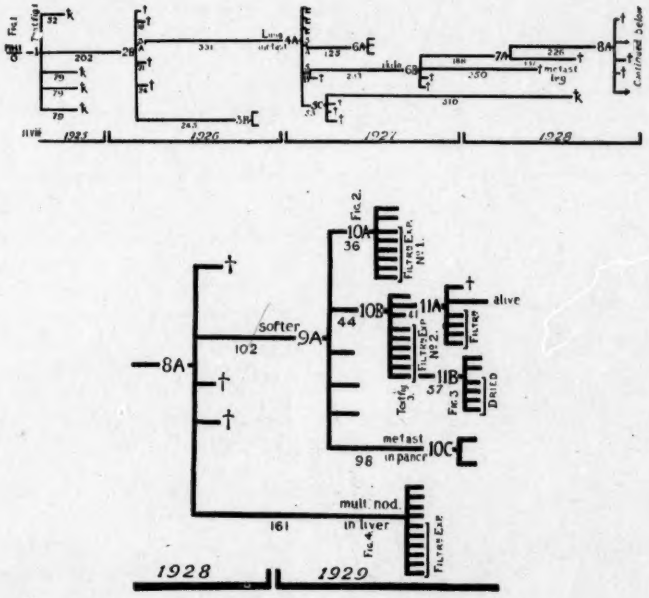
The implantations were always made into the pectoral region on one or both sides. The result of the primary transplantation is shown in Text-fig. 1, and the course of the subsequent propagation in Text-fig. 2. In this and in all subsequent transplantations tumour material was preserved for histological examination so that a complete series of preparations is available illustrating the changes in the structure of the tumour. From these figures and the long intervals (188 to 331 days) between successive passages the extremely slow rate of proliferation characteristic of the growth can be clearly seen. This slow rate of growth and the firm fibrous character were maintained up to the seventh passage.

The lung metastasis mentioned in the preliminary paper (Begg, 1927) occurred in a bird of the third generation (MH 1/3A, Text-fig. 2) which was killed 331 days after inoculation. Another example of metastatic growth occurred on the leg of a bird of the 6th passage (MH 1/6B), which died 250 days after inoculation.

On two occasions, Exp. MH 1/2B and Exp. MH 1/4A, early death of several of the inoculated chicks threatened the loss of the strain and the grafts were

removed, no growth having taken place, and transferred to fresh birds. This transference is indicated in the chart (Text-fig. 2) as a separate passage, although no growth had taken place.

The primary growth presented the microscopical appearance of a hard fibroma. Flattened elongated spindle-cells occur sparsely throughout, between bulky fibrous bundles. The nuclei are flattened elongated ellipses, closely applied to and folded round the connective-tissue strands (Plate—fig. 1). The connective tissue consists of collagen and elastic fibres. The tumour owes its hard elastic character to this admixture of elastic tissue. The blood-vessels



TEXT-FIG. 2.

are well-formed capillaries, forming narrow clefts between the interlacing connective-tissue bundles. In this fissured character the growth resembles the third (XVIII) of Rous' filtrable fowl tumours (Rous and Lange, 1913). It is of interest to note that both these tumours arose from the wall of the gastro-intestinal tract, Rous XVIII from the wall of the gizzard, MH1 from the intestine.

The propagated tumours of the first seven generations maintained the same characters. They were very hard, elastic fibro-sarcomata with varying amounts of elastic fibres among the collagenous fibres.

The first indication of a change in character was noted in a tumour of the 8th transplantation in a bird killed 102 days after inoculation (Text-fig. 2). The growth had only reached a size of 2 × 1·5 cm. but was decidedly softer in

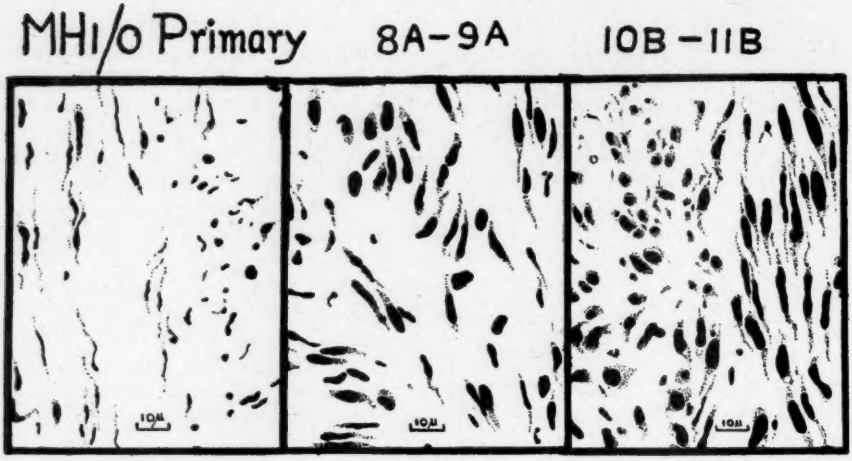


FIG. 1. FIG. 2. FIG. 3.

FIGS. 1, 2 and 3 show the progressively increasing cellularity of the tumour during propagation.

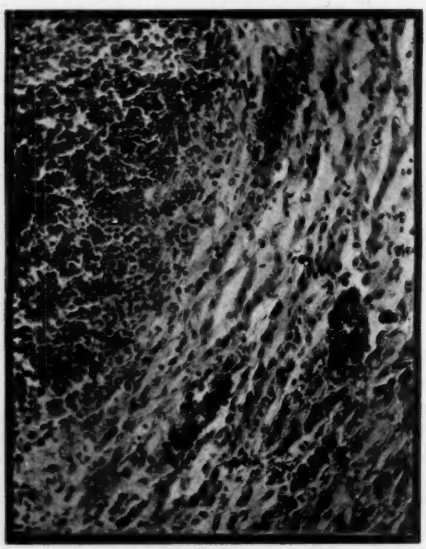
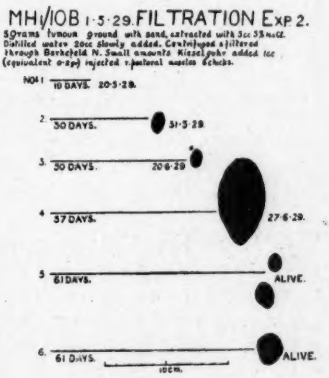


FIG. 4.—Round-celled nodules in liver of a bird of the 8th transplantation. Transplantation and filtration experiments were negative.

FIG. 5.—Photomicrograph of a tumour produced by a filtrate. It shows the cellular character of the tumour and the characteristic fissure capillaries with robust walls.

consistence (Plate—fig. 2). Six chicks were inoculated with this material. The grafts grew more rapidly than the earlier series, reaching a size of 6 × 3 cm. in 36 and 44 days, after which intervals two further series of inoculations were made in the ordinary way and in addition filtration experiments were undertaken.

The tumour, being first ground up with sand, was extracted with 5% salt solution. Distilled water was then slowly added so as to give a final concentration of 1% NaCl. After centrifuging the extract was passed through a Berkefeld candle (N). Six chicks were inoculated in the right pectoral muscles with 1 c.c. of the extract with addition of Kieselguhr in the second filtration experiment (Text-fig. 3). The first appearance of nodules was noted 23 days after inoculation. The result of the second of these experiments, MH1/10B, is shown in Text-fig. 3. In this experiment 80% of the inoculations were positive (5 in 6).



TEXT-FIG. 3.

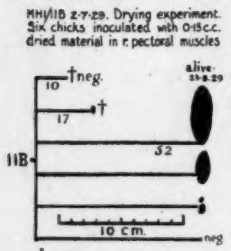
Along with this change in consistence the microscopical appearances show corresponding alterations. The fissured character is maintained, but the tumour-cells are more voluminous and more numerous. The nuclei are larger and less elongated, although still characteristically flattened. Fat droplets occur in the cytoplasm as shown in Plate—fig. 5. The change in histological character is well shown in Plate—figs. 1, 2, 3, in which only the cells with their nuclei are drawn, the spaces between being filled with the fibrous bundles. In the latest, softer tumours, preserved after a shorter term of growth, the elastic fibres are less perfectly formed and do not stain so intensely. Occasionally they appear fragmented into short segments or even rows of granules. Some of the older tumours in the later series are, however, as hard and elastic as those of earlier series.

A tumour from the second filtration experiment, 41 days after inoculation, has been used for propagation. The tumour was of softer consistency. Two chicks were inoculated with cellular grafts and four others with a cell-free filtrate as before. Tumours have developed in 75%.

Another tumour from the second filtration experiment, killed 57 days after inoculation (Text-fig. 2), was minced and dried *in vacuo* over phosphorus pentoxide for five days, the P_2O_5 being changed on the second day. The dry material was powdered and diluted with enough normal saline to give a thick paste. Six chicks were inoculated in the right pectoral muscles with 0·15 c.c. of the paste. Tumours first appeared on the 13th day. The accompanying chart (Text-fig. 4) shows the result of the experiment.

Two further tumours present features of interest. The first of these occurred in a bird of the series MH 1/8A killed 161 days after inoculation. The nodule at the site of inoculation was small and hard. On opening the abdomen multiple white rounded nodules were seen in the liver and spleen, varying in size from that of a walnut downwards. These were soft on section

and healthy throughout. Microscopically (Plate—fig. 4) they consist of closely packed round-cells. The larger masses in the liver have compressed and distorted the columns of liver-cells; the numerous smaller nodules have caused no reaction whatever. The pectoral growth was not transplanted. White healthy nodules were grafted into 4 normal chicks and a filtration experiment was also carried out with this material. All are so far negative. It is therefore improbable that the multiple nodules in the viscera are metastatic from the pectoral growth. A superimposed new growth of another kind seems to be present.



TEXT-FIG. 4.

The second interesting tumour occurred in a bird of the second filtration experiment. It was killed 98 days after inoculation. The very hard tumour in the right breast extended over to the left of the keel. On dissection the growth extended into the thorax and along the hepatic ligament into the abdomen in the region of the pancreas. In the head of the pancreas there was a nodule, 1·5 cm. in diameter, compressing the bile-duct, and causing enormous dilatation of the gall-bladder and biliary stasis so that the liver was deeply stained.

This new growth is an interesting addition to the many recorded filtrable tumours of the fowl. It differs in structure and behaviour from the well-known type Rous No. 1 and from the filtrable endothelioma described from this laboratory (Begg 1927[2]). In structure it resembles rather closely the fissured sarcoma of Rous No. XVIII. In the earlier stages of propagation when it grew very slowly the tumour was so hard that it was impossible to make a filtrate. It is not possible therefore to state definitely whether this character was present from the start, although it is improbable. The subsequent behaviour in becoming softer and growing rather less slowly parallels a condition not uncommon in certain fibro-sarcomata of man and animals.

SUMMARY.

A hard, slowly-growing fibro-sarcoma of the fowl has increased its rate of growth during transplantation and become softer. The histological appearances have changed from those of a hard fibroma to a more cellular and sarcomatous type. It can now be propagated by cell-free filtrates and by dried material, but it has not been proved that it was not so originally.

REFERENCES.

BEGG, A. M.—(1927[1]) *Brit. J. Exp. Path.*, **8**, 147.—(1927[2]) *Lancet*, **2**, 912.
ROUS, P., AND LANGE, L. B.—(1913) *J. Exp. Med.*, **18**, 651.

THE CHEMICAL MECHANISMS EXPLOITED IN THE USE OF MEAT MEDIA FOR THE CULTIVATION OF ANAEROBES.

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Received for publication August 18th, 1929.

THE addition of cooked flesh or other tissue to culture media to facilitate the growth of anaerobes is an old bacteriological device. According to Besson (1895), Roux used sterilized meat hash to grow the *Vibrion septique*. Hibler (1899) tried a variety of other tissues, and found that a *purée* of boiled brain was the most favourable medium, and that no further device to exclude air was required.

Since then a number of bacteriologists have discovered and rediscovered that anaerobes can be propagated in media containing pieces of animal and vegetable tissue, both fresh and boiled, in open test-tubes, notably Theobald Smith (1898), Tarozzi (1905), Harrass (1906) and Wrzosek (1907). The considerable literature on the matter has recently been well reviewed by F. G. Novy, jun. (1925) in his paper on microbic respiration, and also by Hall (1928) in the "Newer Knowledge of Bacteriology."

Although Pasteur (1863) had shown that sterile culture media absorbed oxygen, it was generally supposed that the tissue supplied some nutritive principle which rendered the anaerobe insensitive to the oxygen pressure, until Liefmann (1907) and Guillemot and Szczawinska (1908), by introducing methylene blue into the medium, demonstrated that the cooked meat or other tissue contained reducing systems which removed the oxygen. They pointed out that, owing to the viscous nature of the medium, convection was minimized, and at a depth of 1 cm. below the surface the rate of fixation of oxygen exceeded the rate of supply by diffusion. In the same year Pfuhl (1907) measured the amount of O_2 which disappeared from the air of a flask containing sterilized liver broth.

The intensity of the reducing systems in nutrient broth has recently been investigated by Coulter (1928), Dubos (1929), and simultaneously by Fildes (1929) in his interesting research on the oxidation-reduction potential required to permit the spores of tetanus to germinate.* Coulter measured Eh directly, Dubos and Fildes by means of Clark's indicators. The indicator method pointed to the gradual development of a negative oxidation-reduction potential in the depth of the tubes considerably in excess of that found by Coulter by the electrometric method.

* In this paper Fildes gives a succinct and admirable account of Clark's method of determining oxidation-reduction potential by means of indicators and the essential data required for calculating Eh.

THE REDUCING SYSTEMS PRESENT IN COOKED MEAT.

If a test-tube containing hashed meat of pH 7·5 be autoclaved and allowed to stand the lower portion becomes pink, whereas the superficial part remains of the original brown colour. In the course of months the pinkish stratum recedes to the bottom of the tube. The pink pigment is intimately associated with the coagulated proteins and does not diffuse into the liquid, but when the light reflected from it is examined with a spectroscope the characteristic bands of hæmochromogen are seen. Hæmochromogen cannot exist in the presence of O_2 and, if present, denotes that the medium has a negative oxidation-reduction poten. al considerably in excess of that required to reduce methylene blue.

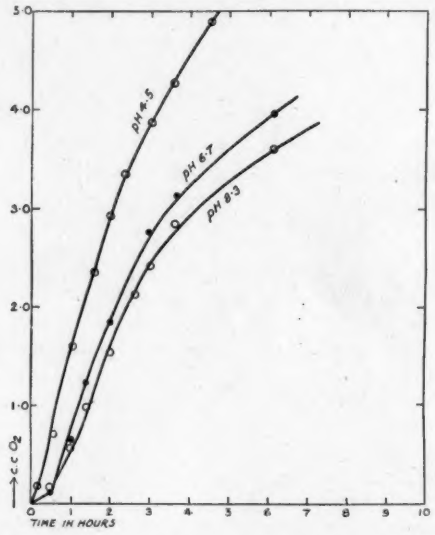

FIG. 1.—Rate of uptake of O_2 per 1 gm. boiled fish muscle (reckoned dry) catalyzed by 1·7 mgm. hæmatin at different pH. t = 37° C.

According to Conant (1923), in order to reduce half the hæmatin present to hæmochromogen at pH 8·5 the addition of enough sodium hydrosulphite ($Na_2S_2O_4$) to produce an Eh of −0·15 volts is required. The reducing power of cooked meat would appear to exceed this for, if indigo carmine is added to a test-tube containing meat at pH 7·5 and autoclaved, this oxidation-reduction indicator is bleached throughout. On exposure to air at laboratory temperature the indicator remains decolorized in the lower half of the tubes for weeks. To completely reduce indigo-carmine at pH 7·5 requires, according to Clark (1923, 1928) an Eh of −0·2 volts. Whether 100% of the indicator is reduced we are not certain as we have not determined the Eh electrometrically. It is also possible that when admixed with other reducing systems having S–H groups, as in culture media containing meat, the colorimetric and electrometric determination of Eh may not coincide.

The pink meat from a culture tube prepared some weeks before was pressed between blotting-paper and 1 gm. suspended in 5 c.c. phosphate solution of pH 7·5. It was placed in a Barcroft differential manometer, and shaken in a bath at 50° C. to prevent the growth of organisms. At the end of 5 days the meat had absorbed 0·45 c.c. of oxygen, *i. e.* about twenty times the volume of O_2 dissolved in the liquid. In this material most of the oxidizable substances had been exhausted during its preparation. As will be shown later, when precautions are taken to avoid this, the uptake of O_2 by cooked muscle is much more rapid.

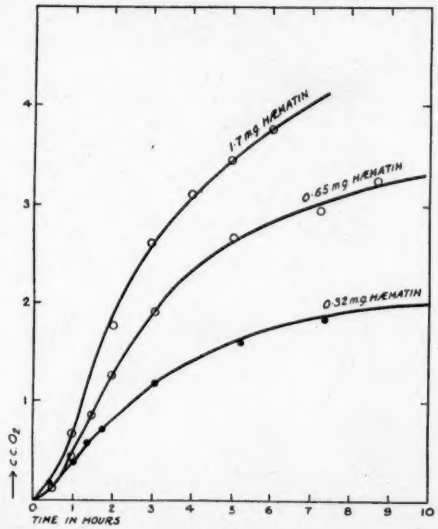

FIG. 2.—Rate of uptake of O_2 per 1 gm. autoclaved fish muscle with varying amounts of hæmatin. t = 37° C.; pH 7·5.

When Hopkins (1921, 1922) discovered the amino-acid glutathione, and that this dipeptide, which is present in muscle, together with some insoluble constituent in muscle, formed a thermostable autoxydizable system, it seemed that a sufficient explanation of the reducing action of cooked meat might be forthcoming.

We found, however, that autoxidation with the co-operation of glutathione is not the only mechanism concerned in the absorption of oxygen by autoclaved meat, for after very thorough washing with hot water, from which the air had been boiled out, it still absorbed oxygen at the same rate when shaken in a Barcroft apparatus at 37° C.

It occurred to us that the hæmatin present in our washed meat, which, as we have pointed out, becomes reduced and forms hæmochromogen, might facilitate the oxidation of some constituent of the meat by acting as an oxygen transporter, as Meyerhof (1923) supposes glutathione does in Hopkins's system.

To acertain whether this was so, we resorted to the muscle of the hake (*Merluccius vulgaris*). When the flesh of this white fish is dissected away from the blood-vessels it is sufficiently free from hæmoglobin and cytochrome for our purpose.

Some samples of this white muscle, after boiling and washing in boiled water, still absorbed oxygen slowly, others not at all, but when a few milligrammes of *a* hæmin, taken up in dilute alkali, or parahæmatin* were added per gramme muscle (reckoned dry) and shaken in a Barcroft's apparatus, the rate of uptake of oxygen was increased more than a hundredfold, indicating that our supposition was correct.

We next extracted the boiled fish muscle with alcohol, anhydrous ether and again with alcohol in a Soxhlet for 6 hours. After this treatment the muscle alone still absorbed oxygen at about the same slow rate as before extraction. The addition of hæmatin now made no difference, but on adding the extract to the extracted muscle in the presence of hæmatin, absorption of oxygen took place as rapidly as with the unextracted muscle. In another experiment a few milligrammes of the waxy ether-extract were emulsified in M/30 phosphate solution of pH 7·5, and shaken in a Barcroft's apparatus at 37° C. both with and without the addition of hæmatin. Absorption of O_2 took place rapidly in the former, slowly in the latter.

There is no doubt, therefore, that the major portion of the oxydizable material is extractable by ether, and in view of the observations of Meyerhof (1923) on the autoxidation of "muscle powder" in the presence of thioglycollic acid, we assumed that it was the unsaturated fatty acids of the lipins which took up the O_2. Neither in Meyerhof's experiments nor in ours was CO_2 formed.

If the material oxidized be the same in the observations of Meyerhof and ourselves, the catalytic influence of hæmatin differs from that of thioglycollic acid in being effective both in alkaline and acid solution. At pH 4·5 the rate of oxidation was 50% faster than at 8·3. At pH 7·5 the rate was intermediate (see Fig. 1). The velocity of the reaction also varies with the amount of hæmatin added (Fig. 2). A preliminary account of these experiments has already appeared (Lepper and Martin, 1928).

At this stage we became acquainted with the observations of M. Robinson (1924) on the catalytic action of blood-pigments in the oxidation of linseed oil. We repeated some of Miss Robinson's experiments, which, we understand from her, were made in an acid medium, and found, further, that the oxidation of this unsaturated oil is catalyzed by hæmatin in alkaline solution as well. In Fig. 3 the rate of uptake at 37° by 20 mgm. of linseed oil emulsified in phosphate solution of pH 7·5 and catalyzed with 0·28 and 0·33 mgm. of hæmatin respectively is shown. The slight catalytic influence of $FeSO_4$ at this hydrogen ion concentration is also shown. The actual amount of Fe added as hæmatin was 1/30 of that added as $FeSO_4$.

The influence of pH upon the rate of oxidation of linseed oil was similar to

* *Note.*—The name given by Keilin to the purplish substance formed by the neutralization of a solution of hæmoglobin to which enough alkali has been added to form "alkaline hæmatin." It was called "kathæmoglobin" by v. Klaveran and neutral hæmatin by Arnold. On reduction it yields hæmochromogen.

that which we had observed with fresh muscle. The rate of uptake of oxygen by an emulsion containing 20 mgm. was about the same as that with 1 gm. muscle (dry weight) under the same conditions, and it was clear that we were dealing with essentially the same auto-oxydizable system that Miss Robinson had described.

Hæmatin has no significant catalytic action upon the oxidation of an emulsion of oleic acid at pH 7·5. At pH 4·5 oxidation proceeded at one-thirtieth the rate observed with a similar emulsion of linseed oil under the same conditions. These results are parallel to those of Meyerhof (1923) with thioglycollic acid as catalyst.

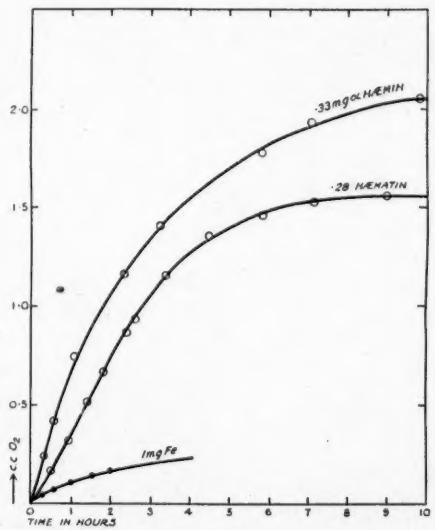

FIG. 3.—Rate of uptake of O_2 at pH 7·5 by 20 mgm. linseed oil catalyzed by hæmatin, parahæmatin and iron. t = 37° C.

We have also made some observations with a specimen of lecithin prepared from egg-yolk, which was kindly placed at our disposal by Dr. Channon, both at pH 4·5 and pH 7·5. In neither case was the rate of oxidation significantly increased by the addition of hæmatin. We were surprised at this result, for Meyerhof (1923) found the oxidation of lecithin from this source to be readily catalyzed by thioglycollic acid. The sample of lecithin we used had an iodine value of 62, and when 1 mgm. of Fe was added oxidation proceeded vigorously for 2 hours. Why the lipins from muscle should behave so differently we are, at present, unable to say.

According to Meyerhof (1923), linseed oil contains about 50% of the glyceride of linolenic acid, so presumably it is the rapid oxidation of this highly unsaturated fatty acid in the lecithins of muscle under the catalytic

influence of the hæmatin present which is principally responsible for the disappearance of oxygen from culture media containing red meat.

The absorption of oxygen by this means proceeds fairly rapidly at 37°C. and is increased in velocity by rise in temperature. Consequently, during the boiling of the meat and its subsequent sojourn in the autoclave at 115°C. all the oxygen is removed. At the same time much of the oxidizable material is exhausted. The experiments from which the graphs in Fig. 4 are drawn demonstrate this. Curve 1 shows the uptake of O_2 by autoclaved red-muscle in which the catalyst is inherent, curves 2 and 3, the difference in

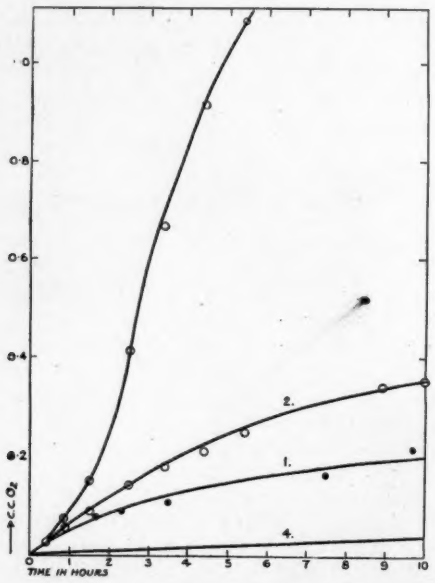

FIG. 4.—Rate of uptake of O_2 per 1 gm. autoclaved muscle (reckoned dry). t = 37° C.; pH 7·5. (1) Red ox muscle from old culture tube. (2) Fish muscle which had been autoclaved in presence of 0·75 mgm. hæmatin. (3) Fish muscle autoclaved and 0·75 mgm. hæmatin added afterwards. (4) Control, autoclaved fish muscle without hæmatin.

the oxygen absorption by white fish muscle according to whether the catalyst hæmatin was added before curve 2 or after curve 3 autoclaving. The other conditions were the same.

Notwithstanding this exhaustion of oxidizable material, however, a residue of unoxidized fatty acids, sufficient to keep all but the surface layer of the medium free from molecular oxygen, remains. The lower portions of the tubes remain free from O_2, for, owing to the viscous nature of the material, the advent of oxygen can only take place by the slow process of diffusion, and a little below the surface the utilization exceeds the supply.

The removal of oxygen through the catalytic agency of the small amount of glutathione present and the considerably more active system—unsaturated fatty acid *plus* hæmatin—are not the only chemical mechanisms present in cooked meat which favour anaerobiosis. The latter mechanism, although potent enough in "removing" molecular oxygen, does not produce the considerable reduction potential observed in our media. Linseed oil mixed with M/30 phosphate solution of pH 7·5 will not reduce hæmatin and form hæmochromagen, nor will it bleach methylene blue, even when exhausted *in vacuo* and kept for months. Extracted fish muscle, on the other hand, readily bleaches methylene blue at this pH and reduces hæmatin to hæmochromagen if the tubes are exhausted of air. It gives a strong nitro-prusside reaction, suggesting that the fixed S–H groups in the proteins, emphasized by Hopkins, are responsible for the reduction potential. If this is the case the S–H groups are curiously insensitive to molecular oxygen, for the nitro-prusside reaction was unimpaired in fish muscle which had been extracted with ether and alcohol and had been kept in a bottle exposed to air for months.

NOTE ON THE PREPARATION OF MEAT MEDIA.

The following method based on experience gained in the course of these experiments is recommended.

Heart muscle is preferable to flesh as it contains about twice as much of lipins. 500 grm. of fresh bullock's heart are minced, placed in 500 c.c. of boiling N/20 caustic soda and allowed to simmer for 20 minutes, by the end of which time the neutralization of the lactic acid will be ensured and the pH of the liquor should be about 7·5. The liquid is drained off on a muslin filter, and whilst still hot, the minced meat is pressed in a cloth and allowed to dry partially by being spread on a cloth or filter-paper. In this condition it can be introduced into test-tubes without soiling them. Enough should be placed in each to occupy about 5 cm. of the tube, and 1% peptone broth, adjusted to pH 7·5, added until the liquid stands 1 cm. above the meat. The tubes must be kept in a bath of boiling water for half an hour to drive off the entangled air, otherwise trouble will ensue when they are subsequently autoclaved at 120° C. for 20 minutes. The inoculum should be introduced towards the bottom of the tube in contact with the pink meat.

SUMMARY AND CONCLUSIONS.

1. The disappearance of oxygen from culture media containing cooked meat is principally due to its energetic absorption by the autoxidation of the unsaturated fatty acids (particularly linoleinic) of the lipins catalyzed by the hæmatin of the muscle.

2. This rapid uptake of oxygen does not occur with the white muscle of a fish unless hæmatin is added.

3. Hæmatin catalyzes the oxidation of free and combined unsaturated fatty acids in alkaline solution as well as in acid solution. In this respect it differs from Fe and thioglycollic acid.

4. The rate of oxidation of cooked meat under the catalytic influence of glutathione is relatively much slower as the content of this dipeptide is so small.

5. The presence of cooked meat in a culture medium of pH 7·5 ensures, if appropriately prepared, a negative oxidation-reduction potential corresponding to an Eh of about − 0·2.

6. The unsaturated fatty acids in the presence of hæmatin remove the molecular oxygen, but do not produce a reduction potential sufficient even to bleach methylene blue ; the glutathione system does.

7. Fish muscle, after thorough extraction with water, alcohol and ether, by which means the traces of glutathione are removed, still gives a strong nitroprusside reaction, bleaches methylene blue and reduces hæmatin to hæmochromagen, and it is concluded that the oxidation reduction potential finally brought about is due to fixed sulphhydryl groupings of the muscle proteins.

8. These chemical mechanisms, one of which rids the medium of oxygen and the other produces a negative oxidation-reduction potential, are admirably suited to bacteriological purposes. The medium is strongly buffered against change of Eh in a positive direction.

9. The fragments of meat pack together in a tube in such a way as to minimize convection. Accordingly, the entrance of oxygen is limited to the relatively slow process of diffusion, and about a centimetre below the free surface the rate of its fixation exceeds the rate at which it arrives.

REFERENCES.

Besson, A.—(1895) *Ann. Inst. Pasteur*, **9**, 179.

Clark, W. M.—(1923) *Rep. U.S. Publ. Health Service* (Reprint No. 84), 1669.—(1928) 'The Newer Knowledge of Bacteriology,' p. 188 (University of Chicago Press).

Conant, J. B.—(1923) *J. Biol. Chem.*, **57**, 401.

Coulter, C. B.—(1928) *J. Gen. Physiol.*, **12**, 139.

Dubos, R.—(1829) *J. Exp. Med.*, **49**, 507.

Fildes, P.—(1929) *Brit. J. Exp. Path.*, **10**, 151.

Guillemot and Mlle. Szczawinska.—(1908) *C.R. Soc. Biol.*, **64**, 171.

Hall, I. C.—(1928) 'The Newer Knowledge of Bacteriology' (University of Chicago Press).

Harrass, P.—(1906) *München. med. Wschr.*, **53**, 2237.

Hibler, E. v.—(1899) *Zbl. Bakt.*, **25**, 513 and 604.

Hopkins, F. G.—(1921) *Biochem. J.*, **15**, 286.

Idem and Dixon, M.—(1922) *J. Biol. Chem.*, **54**, 527.

Lepper, E., and Martin, C. J.—(1928) *J. Soc. Chem. Ind.*, **47**, 1059.

Liefmann, H.—(1907) *München. med. Wschr.*, **54**, 823.

Meyerhof, O.—(1923) Pflüger's *Arch. ges. Physiol.*, **199**, 531.

Novy, F. G., Jun.—(1925) *J. Infect. Dis.*, **36**, 343.

Pasteur, L.—(1863) *C.R. Acad. Sci.*, **56**, 418.

Pfuhl, E.—(1907) *Zbl. Bakt.*, Orig., **44**, 378.

Robinson, M.—(1924) *Biochem. J.*, **18**, 255.

Smith, T.—(1898–99) *J. Boston Soc. Med. Sci.*, **3**, 340.

Tarozzi, G.—(1905) *Zbl. Bakt.*, Orig., **38**, 619.

Wrzosek, A.—(1907) *Ibid.*, **43**, 17.

ON EXPERIMENTAL CARCINOGENESIS: THE LOCAL RESISTANCE OF THE SKIN TO THE DEVELOPMENT OF MALIGNANCY.

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From the Laboratories of the Imperial Cancer Research Fund, London.

Received for publication July 19th, 1929.

WHEN a small area of skin of mice is subjected to tar-painting by applying at regular intervals (twice a week) a drop of tar to the same spot, the action of the tar remains limited to a small area having a diameter of about 4–5 mm. If tarring is discontinued after 4 months one finds then, as was first shown by Bang (1922) and by Leitch (1922), that after a variable interval of time a warty growth appears on the painted area. As judged by inspection and palpation, this growth may be either malignant from the beginning or it may be benign. Histological examination, after operative removal at this early stage, confirms as a rule the diagnosis by inspection of the malignant or benign nature of the growth. If a growth is judged to be benign and allowed to remain without operative interference, it may grow in size without becoming malignant for many months until the animal dies from intercurrent disease; or it may, apparently spontaneously, become malignant. If, however, a large area of skin, about 1 cm. broad and 1·5 to 2 cm. long, is painted with tar for a limited period and then left to itself the picture becomes one of bewildering complexity. In our experiments tar was applied twice weekly for 4 months, when the first growths began to make their appearance. Tar-painting was then discontinued.

THE EXISTENCE IN A TAR-PAINTED AREA OF REGIONS OF VARYING RESISTANCE TO THE DEVELOPMENT OF MALIGNANCY.

The simplest condition is that only one growth appears, which may be either malignant from the beginning, or which may begin as a benign growth and later spontaneously develop into a malignant one. More frequently, however, one observes the appearance of a number of growths all over the painted area. These may all be benign at the beginning until one of them shows a spontaneous development of malignancy, or there may be one malignant tumour from the beginning accompanied by a number of benign growths. If the malignant tumour is allowed to remain it grows, and in the course of its extension merges with the benign growths. But it is rare to find one of the benign growths, remote from the malignant growth, exhibiting a spontaneous development of malignancy, so as to represent an independent focus of a second malignant tumour. In our experiments the few cases in which it occurred were in the later stages when the first malignant tumour has reached a considerable size and occupied the greater part of the area. It

is then difficult to be certain whether the malignant transformation of a papilloma is a spontaneous process, or whether it is due to the infiltration by the first malignant tumour. If one examines closely the malignant tumour, which appears first in its early stages one finds not infrequently, as Deelman (1927) first pointed out, that it arises from two or more closely neighbouring centres, which soon merge into one growth as the two tumours grow. This can sometimes be seen by mere inspection, and can be verified by removing the tumour at an early stage and examining it histologically.

There is evidence that in the development of tar tumours, apart from the local action of the tar on the epithelial cells subjected to chronic irritation, systemic factors play a part. The fact that, other conditions remaining equal, different individual mice vary greatly in the length of the latent period which elapses before a tar tumour develops, has been taken as evidence of the existence of a general resistance. From a consideration of international cancer statistics it has recently been shown (Murray, 1928) that cancer in man is subject to a similar factor of resistance. While the organ incidence of cancer varies greatly for women of different countries, the total incidence of cancer is the same, and if a similar comparison is made for the cancer incidence in men and women of one country the same relationship is found. This conclusion is supported by the experimental observations of various authors that the length of the latent period can be modified, within limits, by various experimental conditions. According to some authors the tar itself has such an action by being absorbed by the skin, and producing changes in the internal organs which are supposed to diminish the general resistance to carcinogenesis. Such an explanation of a general resistance cannot account, however, for the curious condition to which we have drawn attention. There is a relatively large area of skin belonging to the same animal, and therefore subject to the same general systemic factors and to the same degree of local irritation. When malignancy develops in this area from several centres one would expect to find these centres to be irregularly distributed over the whole area. Instead, this development is found to occur with exceptional frequency in one narrowly circumscribed spot. Again, if tar-painting has produced a number of benign papillomata, it is only one, or two closely neighbouring ones, that spontaneously develop malignancy, and not, as one would expect, several of them scattered irregularly over the whole tar-painted area. These findings are significant. They suggest that in an area of skin subjected to chronic irritation there are definite differences in the resistance to the development of malignancy.

THE DEELMAN PHENOMENON.

Three years ago Deelman (1927) published the results of experiments which appeared to give a very striking demonstration of the existence of such a condition. He stated that an incision made in the skin of a mouse in the neighbourhood of a tar cancer leads immediately to the development of a new carcinoma, which appeared in the healing wound. Deelman concluded from his experiments, of which he gave four examples, that the skin in the neighbourhood of a carcinoma is in a precancerous condition, so that the

application of a trauma, insufficient to produce malignancy when applied to the skin elsewhere, will elicit the genesis of a malignant new growth if applied to the skin in the neighbourhood of a tar carcinoma. In view of the importance of these conclusions these observations were repeated in an extensive series of experiments. Both a small area and a large area of skin were painted; different samples of tar were used; the incisions were made inside and outside the painted area; the incisions were made at early and at late stages of tar cancer. More than fifty mice were used in these experiments, but the results were consistently negative. These negative results are confirmed by a recent investigation of Ludford (1929), in which tar-painting was followed by repeated scarification. In these experiments the scarification actually inhibited the development of cancer—a phenomenon which Ludford attributes to the sclerosis of the dermis resulting from scarification. The experiments reported in the following section, in which tar carcinomata were excised in the early stages of their formation without local recurrences in the scar, are further evidence against Deelman's conclusion. One must conclude, therefore, that the results observed by him were due to exceptional circumstances, and that no generalization can be based upon them.

THE SHIFTING OF RESISTANCE TO MALIGNANCY IN A TAR-PAINTED AREA OF SKIN.

If the malignant growth which has first appeared is allowed to remain, it slowly extends over the tar-painted skin and joins up with the other benign growths which may be present in the same area, so that it is impossible to decide whether the appearance of a malignant development elsewhere in the tar-painted skin is an independent phenomenon or is the result of the extension of the first growth, which often grows along the sheaths of the nerve-fibres and forms local metastases in the skin. In order to observe the ultimate fate of the cells in that part of the tar-painted skin which has not developed malignancy, it is necessary to protect them against the invasive growth of the first malignant neoplasm by excising it soon after its malignant character has been recognized. In doing so it is necessary to make a wide excision, so as to be certain that the whole tumour has been excised. If this is done it is rare to get a local recurrence in the scar. Instead, one finds new tumours arising in the remaining area of tar-painted skin, which in view of the wide nature of the excision must have been relatively remote from the excised growth. These new tumours may either be benign papillomata at the beginning which become malignant later on, or they may be malignant from their first appearance. Or lastly, if a papilloma was left behind this papilloma may spontaneously become malignant. The time which elapses between the removal of the first carcinoma and the appearance of the second growth varies greatly. It may be very long, as for instance in an experiment in which a tar-painted area of skin carrying two papillomata and a carcinoma was widely excised. For six months the skin remained apparently quite normal. Then a sessile papilloma appeared near the margin of the excised area. It remained definitely sessile and benign for at least four months, when it gradually became transformed into a carcinoma. A year and a half had elapsed between the

development of malignancy and the beginning of tar-painting. The significance of such an exceptionally long delay will be discussed later.

On the other hand, the interval of time between the removal of the first growth and the appearance of the second may be so short—within 4 weeks

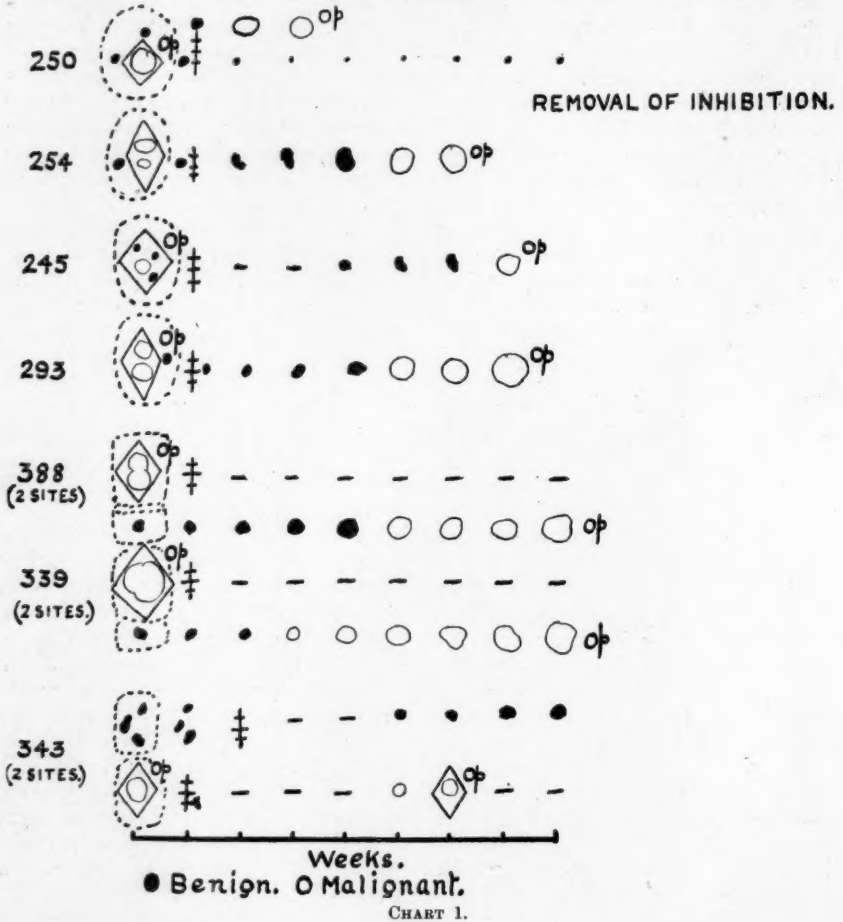


CHART 1.

of the operation—that it is difficult not to correlate the appearance of the second growth with the removal of the first tumour. In a few instances it has been possible to remove the second tumour by operation and to see a third crop arise within a few weeks.

A good illustration of the complexity of the local conditions which determine the development of tar cancer is furnished by the following experiment

Chart 1, mouse 343): Two large areas of skin were painted with tar, one area, which will be called No. 1, between the shoulder-blades, the other, No. 2, on the dorsum of the mouse. After seventeen weeks, when tar-painting was stopped, area No. 1 developed three papillomata which grew rapidly in height but remained benign; three weeks later a small growth appeared in area No. 2, which, when it was excised three weeks after its first appearance, proved to be a carcinoma. The three papillomata were then excised in a successive operation at a week's interval. Within three weeks a new carcinoma had arisen on area No. 2, and a new papilloma on area No. 1. The second carcinoma in area No. 2 was excised after two weeks. Six weeks later a small papilloma developed on this area which underwent spontaneous regression after four weeks. The papilloma in area No. 1, which was allowed to remain, grew, but only very slowly, for several weeks. It then began to ulcerate and eventually regressed. No new tumour had developed when the mouse died from intercurrent disease. A curious feature in this experiment was that the regression of the last two papillomata in the two different areas began at about the same time. Whether this was a mere accident or whether it is of general significance as indicating the existence of a condition of immunity can only be decided by further experiments in which successive crops of tumours in two different areas make their appearance. This is in itself a matter of chance, and there is in addition the technical difficulty that experiments of this kind are necessarily of long duration, and are therefore limited by the difficulty of keeping the animals alive over a sufficiently long period of time.

A very striking result has been observed in cases in which one or several papillomata, together with a carcinoma, were present on the painted area, and in which one papilloma was left behind when the carcinoma was removed by operation. In order to be able to do this without having a local recurrence of the carcinoma, it is necessary that the papilloma should be separated from the carcinoma by a wide enough stretch of skin to allow of a sufficiently wide excision of the carcinoma. It was then found that in a certain number of cases the papillomata began to grow downwards within 4 or 5 weeks after the operation and became malignant. This result was particularly striking in two animals in which the papillomata which had been left behind were very small (Chart 1, mice 254, 293). Sometimes exactly the opposite result was observed: the papilloma disappeared. In one mouse in which two very small papillomata were left behind, separated from each other and from the carcinoma which was excised, one developed into a carcinoma and the other regressed (Chart 1, mouse 250). There were also a number of experiments in which the papilloma which was left behind remained as such for several months and then developed into a carcinoma.

SUMMARY AND SIGNIFICANCE OF OBSERVATIONS ON PARTIAL REMOVAL OF TAR-PAINTED SKIN.

In an investigation of this kind the difficulty is the great variety of the conditions observed in tar-painted mice. In these animals, as in man, the disease is never the same, but varies from case to case. But by observing a sufficiently large number of cases one can detect under this apparently unlimited variety, which is in itself a matter calling for explanation, a definite

sequence of events which occurs sufficiently frequently to be significant. This can best be represented pictographically. The following chart (Chart 2) represents, so to speak, an ideal experiment, which assumes that a number of results which have been observed repeatedly in different mice have actually occurred in one animal. The chart, which explains itself, is thus given as a summary of the experiments detailed above.

It will be convenient now to discuss briefly the significance of these observations. If two mice are painted with tar for the same length of time, and in one a malignant tumour develops after four months while in the second animal it does not make its appearance until the eighth month, this difference is taken as evidence of the difference in the resistance of these two animals to the development of cancer. If in one animal a large area of tar-painted skin shows a malignant development focused in a small part of that area, while the remaining parts show either nothing at all or form benign papillomata, we must postulate differences in the local resistance to the development of cancer. And if, by operative removal of the malignant growth, the area of high resistance is allowed to follow its own course of events and then shows later

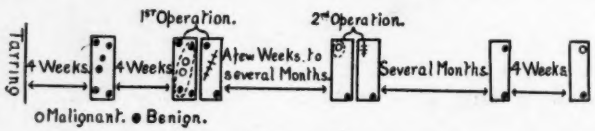


CHART 2.—Pictographic summary.

an independent development of malignancy first in one centre, and after its removal, again in another centre, one must conclude that the local resistance to the development of malignancy is not a fixed property inherent in the tissue, but that it is capable of being varied, and that this variation depends again upon local processes.

The question naturally arises whether the presence of one malignant new growth is in itself capable of inhibiting the malignant development elsewhere in the same area. It is difficult to disregard this possibility in view of the fact that it is rare to find a *simultaneous* development of malignancy in two or three distant centres of the same area, while it is not uncommon to obtain a *successive* development in two or even three distant centres. It is even more difficult not to correlate the development of malignancy in a papilloma immediately after the removal of the malignant tumour with this latter procedure as representing the removal of an inhibition. Lastly, the fact that the period of induction of the second and third crop of malignant growths, as measured from the beginning of tar-painting, is often excessively long may be taken as an indication of the existence of an inhibition.

THE EXPERIMENTAL BREAKING-DOWN OF RESISTANCE TO THE DEVELOPMENT OF MALIGNANCY.

The next stage was to find some other more direct method of demonstrating that a local weakening of resistance to the development of malignancy is an essential condition preceding carcinogenesis. This was achieved by cauterizing

the base of tar papillomata. The experiments were made on mice in which papillomata had been formed as the result of painting a large area of skin. The simplest and hitherto most effective method was to apply a hot needle introduced through a cannula to the base of the papilloma. In another series of animals the skin was incised under ether anæsthesia at a distance of about 1 cm. from the growth; the flap of skin was then raised up so that the base of the papilloma was exposed and a caustic pencil applied to it. Cautery was

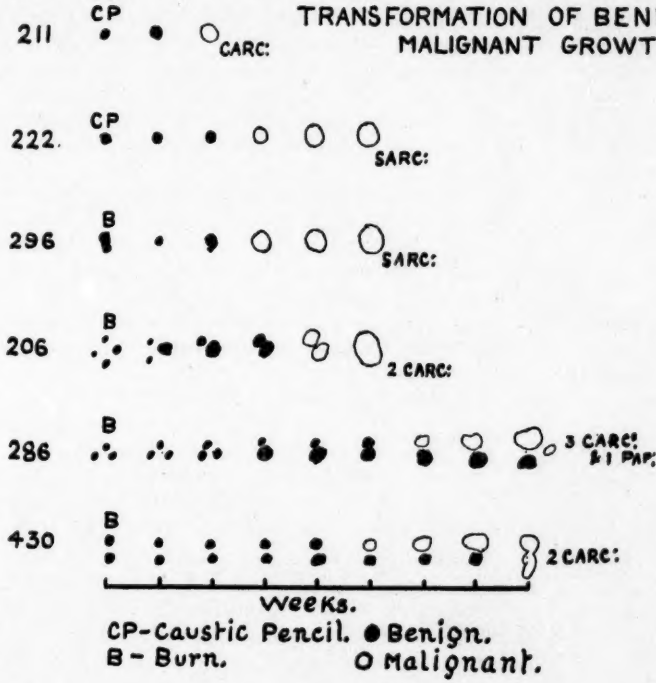


CHART 3.

applied in either of these two ways to papillomata in 14 mice, and of these 6 have developed into malignant new growths within five weeks of the cautery. Their malignant character was verified in every case by microscopic examination. The positive results are represented in Chart 3.

DISCUSSION.

1. *The Analogy to Cancer in Man.*

Some of the phenomena observed in tar-painted mice have their counterpart in the genesis of human cancer. The closest analogy is, of course, presented by the occupational skin cancer occurring in man. In his observations

on paraffin workers' cancer, A. Scott (1923) states that "the usual appearance is that of a gradually growing epithelioma in the midst of a chronic indurated dermatitis with numerous simple warts or indurated warty papules, only one of which has become malignant. I have seldom seen more than one epithelioma at one time on the same individual, though it is not uncommon for the same workman to have different growths at various times over a period of years."

Cheatle's studies (1920) of the early stages of mammary cancer in sections of the whole breast were the first to demonstrate that the malignant process frequently develops in one of several benign growths which are present in the mamma at the same time. From observations on early stages of cancer of the rectum Cuthbert Dukes (1926) has come to a similar conclusion. Both authors emphasize the similarity which exists between these features of the genesis of cancer in man and the experimental carcinogenesis by tar-painting in mice.

Clinical experience presents also an anology for the experiments in which a single burn or a single cautery of the skin elicited *immediately* a malignant new growth. These conditions must be distinguished from those well-known ones where a *prolonged* exposure to heat acting as a chronic irritation produces a carcinoma after the usual long period of induction, as, for instance, in Kangri-cancer. But there are a few cases on record where in man after a single burn or trauma of the skin a carcinoma developed within much shorter periods. The cases are collected in Table I.

TABLE I.

Author and year of publication.	Evidence of potential malignancy and age of patient.	Nature of trauma.	Nature of final tumour.	Interval of time.
1. Story (1885)	No evidence	Carbolic acid	Epithelioma of eye-lid	4 months.
2. Christiani (1892)	Papilloma of skin on forehead in man, aged 36	Bruise	Epithelioma	3 weeks.
3. Langer (1922)	Pigmented wart on back of hand in man, aged 22	Rifle-shot injury	„	1 year or less.
4. Strasser (1917)	No evidence; man, aged 35	Gunshot wound in lower lip	Squamous-celled carcinoma	6 weeks.
5. Pickerill (1926)	No evidence; man, aged 45	Burn	Rodent ulcer	5 months.
6. Ditto	No evidence; man, aged 43	„	Squamous-celled carcinoma	About 2 years.
7. Huguenin (1925)	Ulcer from previous burn; man, aged 35	Burn from hot tar	Spindle-celled carcinoma	1 week.
8. Bang (1923)	Gas worker for 19 years; aged 45	Burn from drop of hot tar in nostril	Cancroid of nostril	2 weeks.
9. Stauffer (1929)	Senile hyperkeratosis of skin of face and neck; man, aged 66.	Burn	Cancroid	4 „

Although the number of cases is small, it will be noted that with two exceptions they have all been recorded during recent years. It is unlikely that similar cases have not occurred in former years. If attention has been paid to such cases only in recent years it is because the recent work on

experimental carcinogenesis has shown that the development of cancer always requires a prolonged application of a trauma and an even longer period of induction. The same is true for occupational cancer in man. In making the comparison between mouse and man one has to consider again that a period of 4 weeks in a mouse corresponds biologically to a period of approximately 2 years in man. Thus the average period of induction of occupational cancer in man is 10–15 years, corresponding to 5–6 months in a mouse. In the table the time which elapsed between the trauma and the appearance of the malignant new growth is much shorter than the usual period of induction in man—in some of the cases it is even much shorter than the period of induction in mice. The cases are particularly instructive because they occurred in the skin, which is an accessible site. The more numerous cases recording the appearance of a malignant new growth, usually a sarcoma, in an internal organ soon after a trauma have always been open to the objection that the neoplasm existed before the trauma, and that the latter merely served to disclose its presence.

The work on experimental carcinogenesis has made it possible to account for a fact which formerly presented a great difficulty to the understanding of occupational cancer—the fact, namely, that cancer frequently develops many years after the individual has left the occupation which is supposed to induce cancer. But now the development of cancer *immediately* after a *single* trauma appears as a new problem which requires explanation, although at first sight the causal connection seems obvious enough. This is the reason why cases of this kind have been recorded only during the last few years. Experimentally it has never been possible to induce cancer in mice immediately after the application of a single trauma. During the last ten years thousands of mice have been subjected to tar-painting and other forms of chronic irritation, and numerous experiments have been carried out with the definite object in view of shortening the period of induction. But no single case has been recorded where a malignant growth developed in a normal mouse within even 4 weeks of the application of a single trauma. We are therefore justified in concluding that in a mouse the *normal* skin does not react to a single trauma by the immediate development of cancer within a few weeks, although the single application of a trauma which acted for a long period has been shown to lead to cancer after a very prolonged period of induction (1 year). In view of the close biological similarity between the development of skin cancer in man and in the mouse, we are also justified in concluding that in those cases in man in which a single trauma was followed immediately by the appearance of a malignant tumour, the skin was not in a normal condition, but in a condition of potential malignancy. Most of the cases collected in the table give evidence of the existence of such a condition as indicated in the table. These cases present therefore a clinical counterpart to our experiments, the trauma effecting a breaking-down of a local resistance to the malignant development of a potentially malignant area of skin.

These considerations have, quite apart from their theoretical interest, a practical bearing on the question of compensation. Let it be assumed that a tar-worker has left his occupation and has become engaged in some other work. As a result of an accidental trauma a carcinoma develops within a few

months at the site of that trauma. It would be a difficult question to decide where the legal liability for compensation lies.

2. *The Conception of Potential Malignancy and its Bearing on Cancer in Man.*

The experiments described in this paper reveal more clearly than was realized before the existence of a condition of "potential malignancy" in a tissue or organ. It means that in a given tissue a large area of cells may be in a condition in which they do not give any manifestation of malignancy either as judged from their histological appearance or from their biological behaviour, but may, nevertheless, be particularly prone to the development of malignancy. Taking mammary cancer in man as an example, one must conclude that the process of chronic irritation which has induced malignancy was not confined to the alveolus in which the carcinoma has arisen, but may have extended—and, if conditions of benign hyperplasia are present, almost certainly did extend—over other parts of the gland .and possibly the whole gland. Even if the malignant growth is not accompanied by benign proliferations elsewhere in the gland, this cannot be taken as a localization of the process of chronic irritation, because in the animal experiments a large area of tar-painting may give rise to only one carcinoma at first, without the appearance of papillomata, but may later, when the first carcinoma is removed, develop a second malignant growth either directly or beginning as a papilloma. The conception of the development of a malignant new growth in any organ or tissue as a local process in its inception remains, but we must now extend this conception by realizing that the appearance of a malignant growth is in itself an indication that the organ or tissue, which is the site of this growth, has been subjected to chronic irritation, and has therefore become potentially malignant over an area much wider in extent than the small area in which the growth has arisen. The extent of this area of chronic irritation which has induced a condition of potential malignancy will necessarily vary in the different organs or tissues. It will depend partly on the form of chronic irritation which has induced the condition, and of this we know as yet little. It will, however, also depend on anatomical and physiological considerations. Thus the area will be relatively small in the cervix of the uterus and very large in the mamma, where the whole organ must always be suspected as having been affected by chronic irritation. It is also likely to be very large in the stomach and the digestive tract generally. When, therefore, a malignant growth has developed in a mamma it is necessary, in the interest of the patient, to treat the whole gland as potentially malignant. Even in the very earliest stages of carcinoma of the mamma a partial removal of the gland, which has recently been advocated again, is therefore undesirable, because the remaining portion may develop a new carcinoma. Clinically, this would be considered as a regional recurrence, and would be ascribed to incomplete removal of the tumour, but it may really be a second neoplasm. Another point of practical importance is that this second development of a malignant growth may occur after a very long interval. In some of the experiments on mice given in this paper 6 months and even a year have elapsed between the removal of the first and the appearance of the second malignant neoplasm. Now a period of

6 months in mice corresponds biologically to a period of 15 years in man. The usual criterion of a cure—survival after 5 or even 10 years—is therefore not applicable where the area of potential malignancy has not been removed. This conception of potential malignancy has an important bearing also on the treatment of cancer by radium. When the area of potential malignancy is very extensive, as in the mamma, destruction of the actually malignant cells by radium may not constitute an adequate treatment. As yet we do not even know whether radium can destroy potential malignancy, and for the reasons given above the usual criterion of 5 years or even 10 years' survival is not applicable. It is possible to decide the effect of radium on potential malignancy experimentally by radiating a tar-painted area, and it is intended to carry out such an investigation. In the meantime it is well to bear in mind in the treatment of early cases of mammary cancer that surgical treatment has the advantage of removing the whole area which is under suspicion.

It may be pointed out that these considerations follow from the facts observed experimentally, and are not dependent on the interpretation given by us that the removal of the first tumour removes an inhibition and thus elicits, or at any rate accelerates, the malignant development in another part of the potentially malignant area.

SUMMARY AND CONCLUSIONS.

If a large area of skin is subjected to chronic irritation by tar-painting, the development of malignancy is confined to a very small portion of that area. Even if the malignant process develops from several centres these centres are almost always situated very close to each other. If benign growths have formed in the area together with a malignant growth the former retain their benign character long after the malignant growth has made its appearance.

If by removal of the malignant growth the area of skin in which no malignant development has taken place is preserved so that its subsequent history can be followed, it is found that in that area malignancy may develop again in a new centre either beginning at the base of a papilloma which was left behind, or starting as an entirely new growth appearing subsequently to the excision of the first carcinoma. Removal of this second malignant tumour has sometimes been followed by the development of a third malignant growth. The interval of time which elapses between removal of the malignant growth and the appearance of a subsequent one varies greatly. Details of experiments are given in which this interval was so short as to suggest a correlation between the two processes. In fourteen experiments in which cautery was applied to the base of a papilloma immediate malignant development was observed in six animals.

These observations taken together supply evidence that the development of a carcinoma is not dependent entirely upon changes in the epithelial cells, but that there are *local* inhibitory factors capable of keeping the malignant development in check, so that the immediate cause of the genesis of a carcinoma may be the *local* removal of an inhibition residing in tissue elements other than the epithelial cells. This resistance is not a fixed property of the tissue

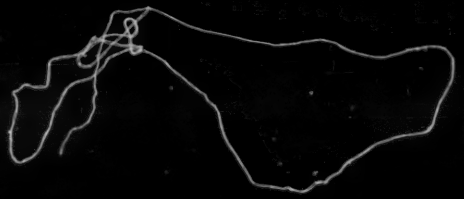
but can be made to shift by experimental interference, and can sometimes be broken down locally by cautery of the tissues underlying a hyperplastic condition of the epithelium. The nature of the tissue elements in the skin which represent this resistance must remain at present a matter for speculation. In view of the shifting nature of this resistance it may be suggested that it is connected with the wandering cells which accumulate immediately underneath the hyperplastic epithelial cells of a skin subjected to tar-painting.

The process of carcinogenesis appears therefore not as a continuous one but as composed of two phases: a process of long duration which induces in epithelial cells the condition of "potential malignancy," which is kept in check by the local resistance of other tissue elements; and secondly a local breaking down of this resistance, which allows of an immediate malignant development of the potentially malignant cells.

The analogy between the experimental observations and clinical and pathological finding in human cancer is pointed out.

REFERENCES.

BANG, F.—(1923) *Bull. Assoc. franç. Cancer*, **12**, 184.—(1922) *C.R. Soc. Biol.*, **87**, 754.
CHEATLE, G. L.—(1920) *Brit. J. Surg.*, **8**, 149.—(1926) *Ibid.*, **13**, 509.
CHRISTIANI, H.—(1892) *Rev. Chir.*, **12**, 57.
DEELMAN, H. T., AND VAN EARP, J. P.—(1927) *Z. Krebsforsch.*, **24**, 86.
DUKES, C.—(1926) *Brit. J. Surg*, **13**, 720.
HUGUENIN, R.—(1925) *Bull. Assoc. franç. Cancer*, **14**, 403.
LANGER, E.—(1922) *Dermatol. Z.*, **35**, 212.
LEITCH, A.—(1922) *Brit. Med. J.*, **2**, 1101.
LUDFORD, R. J.—(1929) *Brit. J. Exp. Path.*, **10**, 193.
MURRAY, J. A.—(1928) *26th Ann. Rep. Imp. Cancer Res. Fund*, London.
PICKERILL, H. P.—(1926) *Lancet*, **2**, 854.
SCOTT, A.—(1923) *8th Sci. Rep. Imp. Cancer Res. Fund*, London.
STAUFFER, H.—(1929) *Z. Krebsforsch.*, **28**, 418.
STORY, J. B.—(1885) *Trans. Acad. Med. Ireland*, **3**, 322.
STRASSER, J.—(1917) *Med. Klin.*, p. 809.



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THE BRITISH JOURNAL OF EXPERIMENTAL PATHOLOGY.

VOL. X, NO. 5. OCTOBER, 1929.

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